UNIVERSITÄT BERN

Graduate School for Cellular and Biomedical Sciences

University of Bern

Evaluation of new concepts for cell based tendon regeneration

PhD Thesis submitted by

Felix Theiss

from **Germany**

Thesis advisor

PD Dr. Peter J. Richards

Center for Applied Biotechnology and Molecular Medicine

Vetsuisse Faculty of the University of Zurich

Accepted by the Faculty of Medicine, the Faculty of Science and the Vetsuisse Faculty of the University of Bern at the request of the Graduate School for Cellular and Biomedical Sciences

Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Science

Bern, Dean of the Vetsuisse Faculty Bern

Graduate School for Cellular and Biomedical Sciences

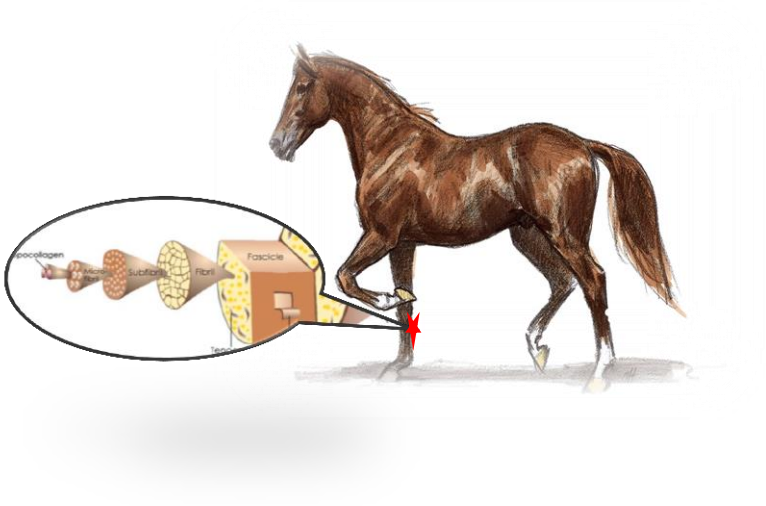
University of Bern

Evaluation of new concepts for cell based tendon regeneration

PhD Thesis submitted by

Felix Theiss

from **Germany**



2015

1 Table of content

2 Thesis Abstract

Background: Understanding and treating tendon injury is of paramount importance in horses and humans. As an alternative to animal models, basic research questions related to tendon injury and healing may possibly be better answered with *in vitro* models utilizing tendon-derived fibroblasts, termed tenocytes. Unfortunately, tenocytes display an unstable phenotype *in vitro* and tend to dedifferentiate when expanded under normal 2-D culture conditions, thereby limiting their usage in tissue engineering and regenerative medicine. By contrast, tenocytes cultured in 3-D systems with different growth factors and mechanical stimuli, have been shown to maintain a more tenogenic-like phenotype. One such 3-D system is the hanging drop culture. This system has the capacity to generate self-assembled 3-D spheroidal microtissues (MTs), which may serve to act as building blocks for tissue engineering applications.

Objectives: The primary objectives of this Thesis were to characterize equine adult tenocytes (EATs), equine fetal tenocytes (EFTs) and equine multipotent stromal cells (MSCs) in the MT format and to evaluate different techniques by which to maintain their differentiation status.

Materials and Methods: MTs consisting of equine tenocytes, MSCs or co-cultures thereof were generated using hanging drop technology and cultured with defined growth media supplements. The tenogenic status of these cells in MTs and respective monolayer cultures were evaluated using histological techniques and through the use of genetic- and protein-based assays.

Results and Conclusions: We could demonstrate that equine tenocytes, MSCs and co-culture thereof formed spheroidal MTs. In contrast to EFTs, which showed increased rates of apoptosis, EATs retained a more differentiated state when cultured as MTs, especially in low serum-containing medium supplemented with TGF-β1, insulin and L-ascorbic acid 2-phosphate, as made evident by increased expression levels of pro-tenogenic markers and tenocyte-like appearance when incorporated into collagen scaffolds. However, the culturing of equine MSCs with tenocytes had no stimulatory influence on MSC tenogenesis or on tenocytes. Taken together, our findings demonstrate the beneficial effects of culturing tenocytes as gravity-enforced microtissue spheroids and supports the use of low-serum containing growth medium in combination with TGF-β1, insulin and ascorbic acid as an effective means by which to maintain the tenogenic differentiation status of cultured tenocytes. This therefore raises the interesting possibility of whether such a system could be directly incorporated into future *in vivo* tissue engineering strategies for the purpose of more effectively treating tendon disorders. Further studies are needed, also to evaluate a possible influence of mechanical stimulation on tenogenic differentiation and for the use of MTs as building blocks for tissue engineering approaches.

3 Abbreviations

ACAN	aggrecan
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
ALSDFT	accessory ligament of the SDFT
ASCs	adipose-derived stromal cells
ASC-MT	MT composed of ASCs
bFGF	basic fibroblast growth factor
BAPN	beta-aminopropionnitrile fumarate
BMA	bone marrow aspirate
BMP	bone morphogenetic protein
BMSCs	bone marrow-derived stromal cells
CABMM	Center for Applied Biotechnology and Molecular Medicine
CAF	cell-activating factor
COL1	type I collagen
COMP	cartilage oligomeric matrix protein
COX	cyclooxygenase
CTGF	connective tissue growth factor
DDFT	deep digital flexor tendon
DCN	decorin
DMSO	dimethyl sulfoxide
EATs	equine adult tenocytes
ECM	extracellular matrix
EFTs	equine fetal tenocytes
EGF	epidermal growth factor
ESCs	embryonic stem cells
FACITs	fibril-associated collagens with interrupted triple helices
GAG	glycosaminoglycan
GDF	growth and differentiation factor
GFP	green fluorescence protein
GM	growth medium
HA	hyaluronic acid
HGF	hepatocyte growth factor
IGF-1	insulin-like growth factor-1

IL-1β	interleukin-1β
IL-1Ra	interleukin-1β receptor antagonist
iPSCs	induced pluripotent stem cells
MTs	microtissues
PDGF	platelet-derived growth factor
PGE_2	prostaglandin E_2
PLGA	polylactic-coglycolic acid
PRP	platelet rich plasma
PSGAGs	polysulfated glycosaminoglycans
ML	monolayer
MMP	matrix metalloproteinase
MSCs	multipotent stromal cells
NGF	nerve growth factor
SCX	scleraxis
SDFT	superficial digital flexor tendon
SL	suspensory ligament
SLRPs	small leucine-rich proteoglycans
TIMP	tissue inhibitor of metalloproteinase
TNC	tenascin-C
TGF-β	transforming growth factor beta
TNMD	tenomodulin
THBS4	thrombospondin-4
UTC	ultrasonographic tissue characterization
VEGF	vascular endothelial growth factor

4 Introduction

Tendon injury is one of the most common forms of musculoskeletal injuries that occur to horses competing in all disciplines. Injuries to the musculoskeletal system have been found to account for 82% of all injuries to racehorses competing in National Hunt and flat racing and of theses 46% involved tendons or ligaments (1, 2). A study by Pinchbeck et al. reported that tendon or ligament strain accounts for 53% of musculoskeletal injuries that occur during hurdle and steeplechase races (3). An epidemiological study over a period of 12 years identified tendon injury as the most common cause for retirement in racing Thoroughbreds in Hong Kong (4). Over a period of one season, 15% of the National Hunt horses (4) and Thoroughbred flat racehorses (5) in training suffered from a tendon or ligament injury. The majority of tendon injuries in racehorses appear in the forelimb (97-99%) (4, 5) with the superficial digital flexor tendon (SDFT) being injured in 75-93% of cases and the remaining injuries occurring to the suspensory ligament (SL) (2, 5). But it is not only in the Thoroughbred race horse population where tendon injuries represent an important economic and well fare issue. Tendon injuries appear in any type of equestrian sport as well as in pleasure horses. Depending on the discipline and thereby the demands on the locomotory system, the incidence of fore- and hind limb involvement as well as the affected tendinous structure varies, with the energy storing tendons (i.e. the SDFT and the SL) being most commonly affected (6-11). The significance of tendon injury is not limited to horses but is also of paramount importance in human medicine. Tendon injuries are reported to represent 30% of the musculoskeletal caseload in a one year study of human general practitioners (12) and tendinopathy is reported to be the cause of 30-50% of all sports-related injuries (13). In humans, tendinopathy is most commonly diagnosed in the Achilles, patellar, rotator cuff and medial/lateral elbow tendons (14-16). Equivalent to the different sporting disciplines in horses, different sporting activities in humans places different tendons at risk. The prevalence of elbow tendinopathy in tennis players can be as high as 40% (17). Due to an increase in the aged population and a rise in participation in recreational and competitive sporting activities, the incidence of tendon injury in humans has risen in recent decades (18, 19). Tendinopathy is a widely accepted generic term that encompasses any abnormal condition of a tendon (20). Still more common in veterinary medicine are the terms tendonitis and tendinosis, also taking preexisting degenerative changes into account.

4.1 Functions of tendon

Tendon and ligaments are connective tissue that guide motion, share loads, and transmit forces in a manner that is unique to each tissue, as well as the anatomical site and biomechanical stresses to which they are subjected (21). Tendons possess different properties depending on function. As in humans, tendons in the horse can be subdivided into two broad categories, the positional tendons and the weight bearing tendons. The positional tendons flex, extend or rotate joints. Weight bearing tendons, such as the equine flexor tendons, located on the palmar/plantar aspect of the equine distal limb (Fig. 1) receive large weight bearing loads because of the hyperextended metacarpophalangeal in the front limb and metatarsophalangeal joint in the hind limb. Weight bearing tendons are more elastic than positional tendons and therefore function as elastic energy stores (22). With the spring-like design of the equine distal limbs, they store energy from weight-bearing for the subsequent stride (23). From the tendons and ligaments positioned on the palmar/plantar aspect of the equine distal limb, this is essentially true for the SDFT and the SL which act as the main energy storing tendons and therefore allow for energy efficient locomotion (24). Additional functions of the flexor tendons and ligaments positioned on the palmar/plantar aspect of the distal limb (termed as the suspensory apparatus) are to support the hyperextended metacarpophalangeal and metatarsophalangeal joint.

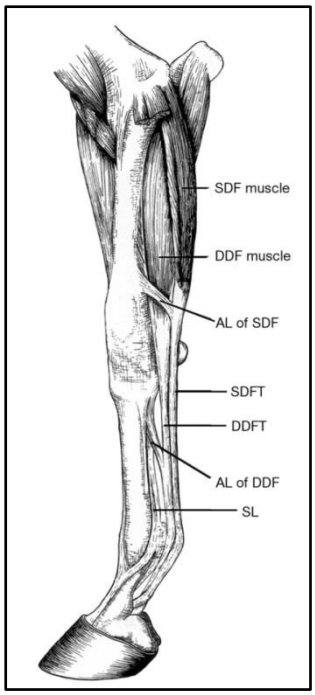


Figure 1: Anatomy of the equine distal limb:
superficial digital flexor tendon (SDFT), deep digital flexor tendon (DDFT), suspensory ligament (SL), accessory ligament of the deep digital flexor (AL-DDF) (23)

4.2 Tendon anatomy and composition

Tendon is a fibrous connective tissue that is composed of cells within an extracellular matrix rich in collagens, proteoglycans, and water (25). The biomechanical characteristics of tendon are determined by the composition and organization of the extracellular matrix. Tenocytes, which constitute 90- to 95% of the cellular elements of tendons, are essential for the formation and maintenance of the extracellular matrix. At least three different populations of tenocytes, based mainly on their nuclear morphology on light microscopy, have been identified in equine tendon and ligament (26, 27). Type I cells with thin, spindle-shaped nuclei, type II cells with more rounded, thick, cigar-shaped nuclei and type III cells, with cartilage-like appearance with round nuclei and visible nucleoli. The proportion of these cells varies between tendon and ligaments, along with tendon site and age. Less mature tendon has considerably larger numbers of type II cells, whereas type I cells predominate with aging. Type III cells are identified in areas subjected to compressive forces (fibrocartilagenous zone). Although the activity of the different cell types is unknown, it is possible that they are involved in mediating different states of extracellular matrix production (22). Tenocytes exhibit long cytoplasmic processes, which link together other tenocytes via gap junctions and thereby allow for communication between cells and appropriate responsiveness to mechanical stimuli (28). The remaining 5- to 10% of the cellular elements of tendons consist of chondrocytes at the bone attachment and insertion sites, synovial cells of the tendon sheaths, and vascular cells, including capillary endothelial cells and smooth muscle cells of arterioles.

The tendon extracellular matrix is composed predominantly of water (about 65% of wet weight), collagen (about 30% of wet weight) and non-collagenous glycoproteins (about 5% of wet weight). Collagens and proteoglycans account for 70-80% of the dry weight, including small leucine-rich proteoglycans (29). The fibril forming type I collagen represents about 95% of the total collagen. The remaining 5% are made up mainly of type III and V collagens. In contrast to the mid-substance of the tendon, type II and III collagen are predominant in the fibrocartilaginous zone, where only minor amounts of type I, IX and X collagen are present (21).

The hierarchical structure of tendon and its functional properties are determined by the collagen types present, as well as their supramolecular organization (Fig. 2). The fibril is the smallest tendon structural unit consisting of collagen molecules. Bundles of fibrils are organized as fibers, which are grouped together with tenocytes as fascicles. Fascicles are surrounded by a cellular, loose connective tissue, the endotenon. In relaxed tendon, the fascicles have a waveform known as crimp, which is partly responsible for tendon elasticity. The endotenon carries blood vessels and nerves and contains a different type of cell. These more rounded cells are believed to represent a source of multipotent

stromal cells (MSCs) within the tendon (30). Bundles of fascicles are enclosed by the epitenon. More superficially, a third layer of connective tissue called the paratenon surrounds the tendon. Epitenon and paratenon, both reducing friction with the adjacent tissues, are termed peritendon (31).

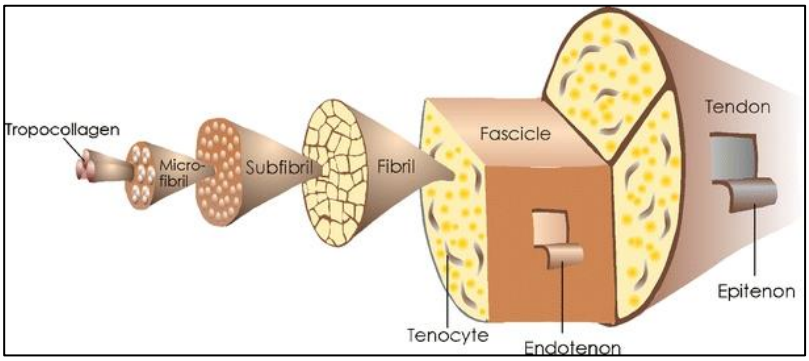


Figure 2: The organizational structure of tendon (32)

Two classes of regulatory molecules are involved in the regulation of linear and lateral fibril growth, the small leucine-rich proteoglycans (SLRPs) and the fibril-associated collagens with interrupted triple helices (FACITs). From the SLRPs, two sub-classes exist. In tendon, decorin (class 1) and fibromodulin (class 2) are dominant in this regulation, but can be modulated by biglycan (class 1) and lumican (class 2). The FACITs closely interact with the fibril forming collagens and affect surface properties as well as fibril packing (21). The FACITs XII and XIV have been identified during tendon development (33, 34). Cartilage oligomeric matrix protein (COMP) is also thought to be involved in in the organization of the collagen fibril framework during normal growth and development. Experimental data have shown that COMP accelerated collagen fibril formation *in vitro* and that levels of COMP increase dramatically during growth and peak at around 2 years of age in the tensional region of the equine digital flexor tendons (35). The importance and relevance of this protein is further suggested by a correlation between ultimate tensile strength of the tendon and the level of COMP at skeletal maturity (36). However, knockout studies in COMP-null mice have not found any tendon abnormalities (37). The collagen fibrils themselves, as well as the covalent intra- and interfibril collagen cross links and electrostatic cross links provided by non-collagenous proteins, contribute to the tendon´s biomechanical properties (22). Furthermore elastic fibers are a prerequisite for the elastic modulus of tendon (38).

4.3 Epidemiology of tendon injury

Overstrain tendon injuries have been shown to significantly impact on the athletic performance and significantly increase morbidity and mortality rates in racehorses (1, 2, 5) as well as in horses performing in other sporting disciplines (6-10). Several studies in racehorses are available, which identify the SDFT and second the SL to be the most prevalent injured structure (2, 5) with the forelimbs being 4-5 times more likely to be affected (1). In a study performed by Trump et al. (11), 1527 cases of non-racehorses, which were examined in a referral hospital because of injuries to the SL, the digital flexor tendons and associated structures were evaluated according to their athletic type of use. The SL was the most common affected structure in pleasure horses, dressage horses and show jumpers. The SDFT was most commonly affected in eventing horses. When evaluating front and hind limbs separately in pleasure riding horses, the most commonly affected tendons in the front limbs were the SDFT, the SL and the accessory ligament of the deep digital flexor tendon (AL-DDFT). However in the hind limbs, the SL was far more commonly affected than the SDFT and deep digital flexor tendon (DDFT). In dressage horses, the SL was by far the most commonly affected structure in both front and hind limbs. The second and third most commonly affected structure in the front limbs were the SDFT and the AL-DDFT. In show jumpers and in eventing horses, the SDFT was more often affected than the SL in the front limbs, whereas the SL was the more commonly affected structure in the hind limbs. The comparative risk for a forelimb to be affected compared to a hind limb was 3.0 times higher in eventing horses, 2.6 times higher in show jumpers, 1.8 times higher in dressage horses and 1.7 times higher in pleasure horses. Even though different equestrian disciplines place different demands on the locomotory system of the horse, these data highlight the fact, that the major energy storing tendons, i.e. the SDFT and the SL, are more prone to injury than any other tendon structure within the equine distal limb (30).

4.4 Mechanisms of tendon injury

Tendons can suffer from intrinsic (strain) and extrinsic (percutaneous) injury. Extrinsic injuries are relatively rare, and are associated with skin lacerations in the majority of cases. Overstrain injuries are believed to appear by one of two mechanisms. They can result from sudden overloading of the structure, which overwhelms its resistive strength. However, for most of the strain-induced injuries in the horse involving the palmar/plantar tissue structures of the metacarpus/metatarsus, clinical injury is believed to be preceded by a phase of molecular degeneration. Cumulative fatigue damage

weakens the tendon matrix and allows the initiation of clinical tendonitis when submaximal loading overcomes the resistive strength of the tendon.

Several reasons are discussed in the literature as to why the SDFT and the SL are prone to injury and how degenerative changes may predispose the tendon to injury. Normal strains in the SDFT recorded *in vivo* are in the region of 3% to 8% at the walk, 7% to 10% at the trot and 12% to 16% in the gallop (39). Maximal strain (i.e. percentage elongation) to failure (rupture of the tendon) in the SDFT ranges from 12 -21% *in vitro* (40-42). These data underline the concept that at maximal exercise, the SDFT operates close to its functional limits with a narrow safety margin, and thus only minor influences may result in tendon injury (43). A study in ponies has shown that the SDFT and the SL are subjected to almost the same level of loading during walk and trot exercise *in vivo* (44). As the ultimate tensile force needed to rupture the SL is higher than that for the SDFT (45), the SL may have a larger tolerance at faster gaits when peak loads occur.

In contrast to the SDFT, the primary function of the DDFT appears to be flexion of the distal phalangeal joint during the late swing phase of the stride, rather than storage and return of energy (46). Correspondingly, *in vivo* studies have shown that the DDFT experiences lower peak forces and strains during locomotion than the SDFT and the SL (44, 47). The muscle of the DDFT contains a high percentage of fast twitch muscle fibers and therefore is more susceptible to fatigue. As the DDFT stabilizes the metacarpophalangeal joint during hyperextension, fatigue of its muscle destabilizes the metacarpophalangeal joint and therefore the SDFT will be subjected to higher strains (47). Several studies have indicated a higher risk of tendon injuries at the end of a race, when racing over longer distances or over fences (1, 3, 48, 49).

The strength of a tendon is correlated to the number and size of the collagen fibrils. Collagen fibrils increase in size during development and in response to increased physical demands or training (32). Intermolecular covalent crosslinks between fibrils also play a role in the tensile strength of tendon. The conversion of chemically reducible divalent crosslinks to a network of non-reducible trivalent crosslinks is thought to be responsible for a large proportion of the increase in the mechanical strength of tendons as they mature. It has been hypothesized by some authors that the mid-metacarpal region of SDFT in the horse can adapt to exercise during skeletal development, but is has little or no ability to do so after skeletal maturity (5, 50). Patterson-Kane et al. were able to show that the SDFT and DDFT are mature by two years of age, being made apparent by the replacement of immature collagen cross links with no further increase in collagen fibril size and cessation of rapid changes in the collagen fibril crimp morphology (51).

Epidemiological studies have supported a strong association of age and exercise with the incidence of tendon injury in horses (52) and people (53). Direct low-grade mechanical forces around the yield point (of the stress-strain curve) led to cumulative fatigue micro-damage of the tendon matrix. In long term exercised horses and in aged horses, the non-collagenous component of tendon shows changes in the center of the tendon where clinical injury is seen and where there is an accelerated loss of glycosaminoglycans (GAGs) and COMP (30). Interestingly another study found increased levels of GAGs, as well as increased levels of type III collagen and cellularity in the discolored central core region of degenerated superficial digital flexor tendons (54).

Regional differences in collagen fibril diameter in long term exercised older horses, but not in short term exercised or younger horses, have also been detected (55). This higher proportion of shorter fibrils within the central region of the SDFT in comparison to controls is assumed to result from disassembly of larger diameter fibrils. Additional exercise-accelerated degenerative changes that occur inevitably with ageing represent a regional reduction of the collagen crimp angel and length in the central core of the tendon (56, 57). Under loading conditions, central fibers are straightened first, and hence are most likely to rupture first. Changes in matrix composition, a smaller fibril diameter and a reduction of the collagen crimp angel and length are mainly seen in the central core area of injured tendons. This offers a possible mechanistic explanation for the generation of core-lesions frequently seen in clinical cases of tendon injury.

Changes in matrix composition present in degenerated tendons suggest an increased rate of matrix turnover, which may be an inadequate healing response or an inappropriate cellular response. In addition to mechanically induced micro-damage, other etiologies have also been described including hyperthermia, hypoxia or, in human tendons, neurogenic (58). Hysteresis refers to the energy loss between the loading and unloading cycle of tendon determined from the area between these two curves. Hysteresis is usually about 5% in equine tendon (40). Much of this energy is lost as heat and results in a temperature increase within the core region of the tendon. In galloping horses, temperatures of up to 45°C have been detected and are suggested as a causative factor for tendonitis of the SDFT (59). *In vitro* experiments have shown that tenocytes are much more resistant to these temperature increases in comparison to other fibroblast-like cells (60). However, another study has shown increased production of pro-inflammatory cytokines in equine tenocytes at temperatures above 45° C, which will likely increase the production of matrix degrading enzymes (61). Therefore, hyperthermia may still influence tendon matrix quality. The relative poor blood supply in tendons limits heat dissipation but may also result in low oxygen levels within the core

region of tendons. Low oxygen supply may compromise the synthetic and degradative activity of tenocytes.

Furthermore, the upregulation of matrix degrading enzymes, through over- or under-stimulation of tenocytes, may also result in degenerative changes. Several *in vitro* studies have demonstrated that tenocytes under cyclic stretching express matrix metalloproteinases (MMPs), interleukin-1β (IL-1β) and prostaglandin E_2 (PGE_2) (62-64). These studies suggest that the strains experienced by the SDFT may cause cells to produce proteolytic enzymes, which will break down the matrix. In a study performed by Clegg et al. (65), *MMP13* gene expression was elevated substantially in acute and chronic tendon injury, whereas *MMP1* gene expression was just elevated in acute injury. As in studies in man (66, 67), *MMP3* gene expression was decreased in acute and chronic tendon injury. *ADAMTS4*, *TIMP1* and *TIMP2* levels were unchanged compared to normal tendon, whereas *ADAMTS5* and *TIMP3* levels were substantially decreased in acute tendon injury. As MMPs are tightly controlled at a number of levels, including synthesis, secretion, activation of the zymogen, localization and clearance of the enzyme, such studies do not necessarily reflect the active proteinase profile within the tissue. Furthermore they also have important roles in normal physiological events in tendon homeostasis and repair. Alternatively, stress shielding of cells due to micro-damage, leading to unloading of the damaged fibrils, will alter the cell-matrix interactions. Stress deprivation causes increases in expression levels of the collagenase *MMP13*, alterations in cell morphology and pericellular environment (68), and increases in apoptosis (69).

4.5 Clinical presentation and diagnosis of tendon injuries

Clinical tendonitis varies in severity, from individual fibril or fiber slippage, to individual fibril or fiber rupture, and ultimately to complete rupture of tendon with progressive involvement of more groups of fibers and fascicles. Clinical signs of tendon injury vary considerably depending on the affected structure, location of injury within a certain structure, severity and the timing of examination (i.e. time from onset of injury and thereby stage of tendon healing). Furthermore, a lack of correlation may exist between the severity of injury and the clinical signs, especially lameness present, more likely representing the degree of inflammation rather than the degree of damage (30). Depending on anatomical location, enlargement of the affected structure may be less obvious in deeper, less superficially positioned structures. In acute tendon and ligament injuries nearly all cardinal symptoms of inflammation are present. An increase in surface temperature can be the earliest and most subtle clinical sign of injury or re-injury detectable by digital palpation, in more superficially

located structures or thermography. Sensitivity to direct digital palpation, best done in comparison to the contralateral limb, further confirms the affected structure and location. Depending on location of the tendon injury, enlargement of the affected tendon may be detectable by digital palpation or by macroscopic evaluation of the tendon profile (Fig. 3a). As only enlargement of a tendon may also be the end-stage of repair of a previous tendon injury, it should be considered along with other inflammatory symptoms in more acute cases. Even though not specific to tendon injury, it is accompanied by increased subcutaneous or peritendinous fluid accumulation, detected macroscopically by the less defined appearance of subcutaneous positioned structures and again, by digital palpation. The degree of lameness varies between affected structures, location within one structure, time from onset of injury, severity of injury, type and athletic use and in between individuals. In general terms, more acute and severe injuries carry a higher risk of severe lameness. In more chronic and mild cases of bilateral tendon injuries as seen in SL desmitis, lameness may not be an obvious sign of injury but reduced performance or the ability to shift the weight to the hind quarters may be the only clinical symptoms. Complete rupture of the digital flexor tendons or the SL is generally accompanied by severe lameness, even though improving relative quickly over time. Through the loss in support, complete rupture of the SDFT or the SL leads to pathological overextension of the metacarpo-/ metatarsophalangeal joint. In cases of complete rupture of the DDFT, the toe of the hoof rises during the stance phase of the affected limb. In certain anatomical locations where tendons are enclosed by synovial sheaths, i.e. carpal-/tarsal sheath and the digital flexor tendon sheaths, tendon injury involving the outer boarders of the tendon lead to tenosynovitis, characterized by increased filling of the sheath.

The most common imaging modality to confirm a tendon injury, but also to objectively asses the severity of injury and to track the stage of tendon healing, is diagnostic ultrasound (Fig. 3b). Cross section area of the tendon and defect zone, length of the defect, echogenicity of the defect zone in comparison to healthy tendon and fiber alignment during the stages of healing are classical parameters recorded. An advancement of the classical ultrasound, even though not widely used, is the computerized ultrasonographic tissue characterization (UTC), a system consisting of both hardware and software for quantitative evaluation of tendon structural integrity. In UTC, a precision instrument along the long axis of the tendon drives a high frequency ultrasonographic probe. Transverse images are stored at real time for later processing by compounding contiguous transverse images into a 3D ultrasonographic data-bloc, which can be used for tomographic visualization, tissue characterization and calculation of lesion size. In selective cases where the suspected injury zone is

inaccessible for ultrasound, as in the case of DDFT injury within the hoof capsule, magnetic resonance imaging is the diagnostic modality of choice.

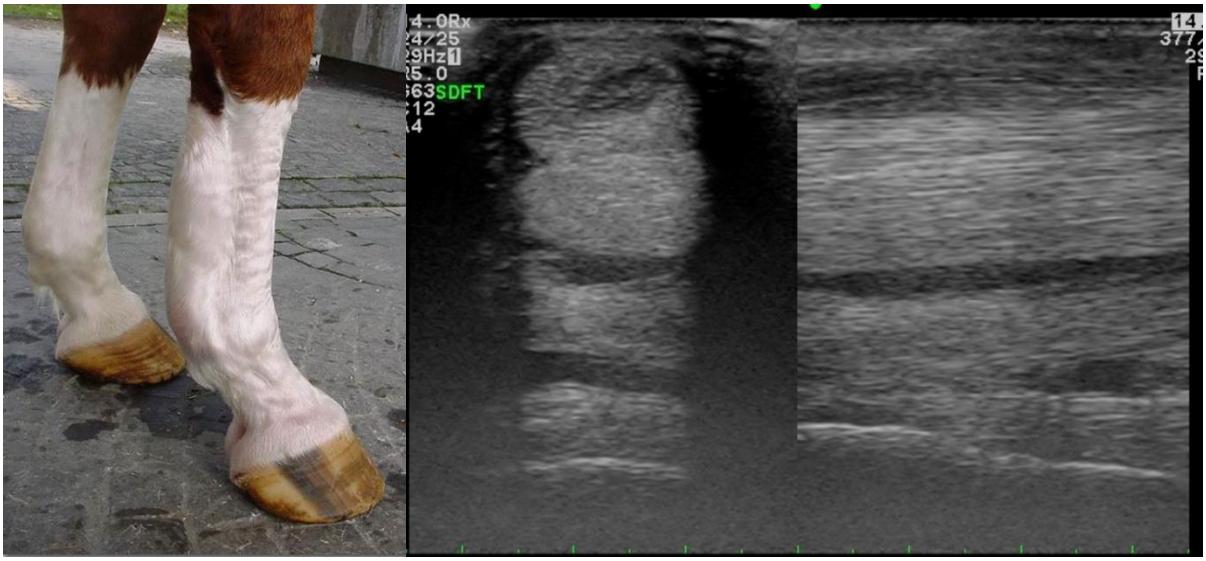


Figure 3a: Macroscopically enlarged SDFT

Figure 3b: Ultrasonographic image of SDFT tendonitis

4.6 Tendon healing

Once the peak load on the tendon overcomes its structural strength, there is physical damage of the tendon matrix. The damage induces as repair process similar to that found in other soft tissues, such as skin, characterized by inflammation followed by fibroplasia. With the onset of clinical injury, the acute inflammatory phase begins, in part determined by the severity of the injury and the anti-inflammatory therapy initiated. The inflammatory phase is characterized by intratendinous hemorrhage, increased blood supply and edema, and infiltration of leukocytes, initially neutrophils, followed by macrophages and monocytes. In general, the inflammatory phase lasts 1 to 2 weeks. The sub-acute reparative or fibroblastic phase starts a few days after the onset of injury, overlapping with the inflammatory phase and peaks at around 3 weeks. It is characterized by a strong angiogenic response and the accumulation of fibroblasts within the damaged tissue, which are responsible for synthesizing the scar tissue. The morphology of the invading fibroblasts differs from that of normal tenocytes. They are larger and more basophilic and have large vesicular nuclei, and are thus more similar to myofibroblasts than tenocytes (70). The chronic remodeling and maturation phase begins several months after the injury. The newly formed collagen in the scar is not as highly cross-linked as in normal tendon. Type III collagen, present in low amounts in normal tendon (< 1%), is as high as 20 – to 30% in tendon scar tissue (70). The significantly greater proportion of type III collagen is believed to deleteriously affect the mechanical properties of the tissue (71-74). The previously formed scar

tissue is remodeled over the course of several months, being identified by improved fiber alignment and conversion of type III collagen to type I collagen (75-77).

To achieve sufficient structural strength, larger amounts of fibrous tissue are laid down within and around the tendon, giving rise to a tendon that is persistently enlarged but which also has greater structural stiffness (Fig. 4). Furthermore, the healed tendon fails to retain the biomechanical properties of the original tendon prior to the injury (78) due to persistently deficient structural organization and composition of the matrix (Fig. 4) (70). A significantly greater proportion of type III collagen is just one of the factors contributing to the sub-standard mechanical properties of the tissue (71-74). The increase in stiffness reduces the efficiency of the tendon as a spring and compromises the performance of the horse (43). Furthermore, the increased stiffness in the repair tissue results in increased strain rates in adjacent and relatively undamaged regions of the tendon, which are therefore more prone to injury. Besides the insufficient healing response of tendon tissue, adhesion formation between tendons and adjacent tissue further compromises function of the healed tendon. The hypocellular histological structure, poor blood supply and bradytrophic metabolism of tendons are thought to be major reasons for their limited self-healing properties (79, 80).

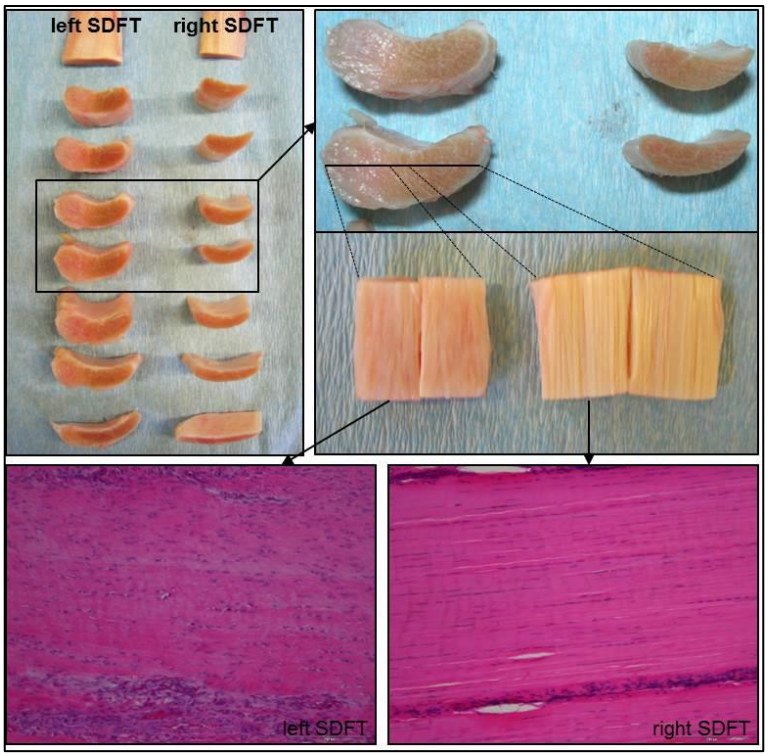


Fig. 4: Tendon healing one year after SDFT tendonitis in the left front leg of a 11-years old eventing horse; the left SDFT is macroscopically enlarged and the defect area still displays discoloration. Histologically, a higher cellularity can be seen along with a less well organized ECM and increased numbers of blood vessel.

4.6.1 Cells involved in tendon healing

Cells involved in the repair process may originate from different sources within the tendon (e.g. resident tenocytes, endotenon and paratenon cells) and from the peripheral circulation. Therefore, some authors differentiate intrinsic (81) and extrinsic (82) repair in tendon damage (83, 84). Wong et al. have demonstrated that flexor tendon healing is more complex than can be accounted for by the intrinsic and extrinsic concepts alone (85). It is now believed that these two mechanisms act cooperatively. It is hypothesized that first, fibroblasts and inflammatory cells from the tendon periphery, blood vessels and circulation are recruited to the injury site, thereby contributing to cellular infiltrate and the formation of adhesions. Thereafter, intrinsic cells from the endotenon are activated as they migrate and proliferate at the injury site, recognizing the ECM and giving support to the internal vascular networking (86, 87). The origin of the reparative cells remains in debate. Using a model of tendon injury in two different chimeric rats, where one group expressed green fluorescent protein (GFP) in circulating mesenchymal cells and the other group expressing GFP in the patellar tendon, Kajikawa et al. were able to show an initial invasion of circulating mesenchymal cells, followed by the activation of local cells, which participate in the proliferative phase and carry out the remodeling phase (88).

4.6.2 Growth factors involved in tendon healing

Several growth factors and cytokines are involved in tendon healing (89, 90). The most investigated cytokine is transforming growth factor- β (TGF-β) (91). TGF-β belongs to a family of related proteins that regulate many cellular processes. Mammalian TGF-β has three known isoforms (TGF-β1, - β2 and - β3) and in many instances these isoforms are indistinguishable in their effect on cell behavior. All three isoforms are believed to signal through the same TGF-β receptors (TβRI, TβRII and TβRIII) and are secreted as latent precursor molecules that require activation, thereby allowing the TGF-β ligand to bind to its receptor and activate the intracellular pathway. TGF-β1 is upregulated in the flexor tendon/synovial complex in the early postoperative period (92, 93), with TGF-β receptors being upregulated after tendon injury and found concentrated along the repair site (94). Immunohistochemistry and Western blotting showed low levels of TGF-β in normal, healthy equine tendon, but after tendon injury higher levels of TGF-β were detected around the injury site (95). In tendon injury, TGF-β recruits fibroblasts and macrophages to the site of injury and induces increased proliferation, angiogenesis and up-regulation of MMP inhibitors and collagen expression, as well as downregulation of proteinase activity (96). However, its upregulation also has implications for

pathological scar formation (97). Connective tissue growth factor (CTGF) is a downstream mediator of TGF-β1 signaling and acts as a co-factor in fibrosis (98). In an experimental model, elevated gene expression of both *CTGF* and *TGF-β1* was observed in early tendon healing (99). Hepatocyte growth factor (HGF) secreted by mesenchymal cells, activates its biological activity after proteolytic cleavage through its receptor c-Met. In an Achilles tendon model, HGF was implicated as inhibiting TGF-β1-induced fibrosis by reduction of type III collagen expression and decreased expression of α-smooth muscle actin (100). Fibroblast growth factors (FGFs) are a family of related polypeptides. Basic fibroblast growth factor (bFGF/FGF2) is found in early wound healing and is known to have a role in fibroblast chemotaxis, proliferation and angiogenesis. It has also been shown to be present in uninjured as well as repaired flexor tendons (101-103), even though its role in tendon healing remains unclear. *In vitro* bFGF causes tenocyte proliferation and type I collagen synthesis (104). Transfection of bFGF into damaged and repaired tendons increased tendon healing and strength (105). Furthermore Thomopoulos et al. found increased vascularity and cellularity in healing flexor tendons treated with bFGF (106). Insulin-like growth factor-1 (IGF-1) is one of three single chain polypeptides belonging to the insulin-like growth factor family (IGF-1, IGF-2, Insulin). Beside its role in wound healing, *IGF-1* is expressed in uninjured flexor tendons (107) and is upregulated after tendon injury and repair (99). *In vitro* IGF-1 and IGF-2 stimulated proteoglycan, collagen and non-collagen protein synthesis in flexor tendon-derived cells (108). In another *in vitro* study by Herchenhan et al., IGF-1 supplementation of engineered human tendon tissue imparted a stimulating effect on fibril diameter and the expression of several genes including collagen, tenomodulin (*TNMD*) and scleraxis (*SCX*) (109). In a collagenase-induced model of flexor tendonitis, intralesional injection of recombinant human IGF-1 improved cell proliferation and collagen content as well as a trend toward increased stiffness of healing tendons in horses (110). Vascular endothelial growth factor (VEGF), present in several isoforms, shares around 20% amino acid homology with platelet derived growth factor (PDGF) (111). Their biological activity is exerted over three tyrosine kinase receptors, and the bioavailability depends on the isoform binding to the receptor. The up-regulation of mRNA expression of *VEGF* during the early stage of flexor tendon repair suggests some type of involvement. In an Achilles tendon model, *VEGF* gene therapy increased *TGF-β* gene expression and exogenous VEGF appears to increase tensile strength (112). VEGF is an important angiogenic factor, which plays a role in tendon rupture, healing and development (113).

Bone morphogenic protein (BMP) and growth and differentiation factor (GDF) belong to the same superfamily as TGF-β. Several BMPs/GDFs appear to play a role in tendon biology. Animal models with genetic mutations of *BMP14* (*GDF5*), *BMP13* (*GDF6*) and *BMP12* (*GDF7*) have shown altered

Achilles tendon phenotype (114-116). GDF8 (Myostatin) has been shown to be expressed in tendons. Mice deficient in GDF8 have weaker, hypocellular, and more brittle tendons (117). BMP12 enhanced tendon healing by increased synthesis of type I and III collagens, resulting in increased tensile strength (118, 119). In an Achilles tendon transection model, BMP12 was highest at 1 week and declined over the 8 weeks study period (120). Lee et al. successfully utilized BMP12 in order to differentiate MSCs into tendon-like tissue for the purpose of tendon reconstruction (121). In a study by Tan et al. BMP14 improved tendon repair, tenogenic differentiation, increased scleraxis and the ratio of type I to type III collagen in MSCs (122). Epidermal growth factor (EGF) plays an important role in regulating cell growth, proliferation and differentiation through its receptor EGFR. Tsubone et al. showed increased expression of EGF after canine flexor tendon repair in inflammatory cells surrounding the repair site (93). Platelet derived growth factor (PDGF) is a dimeric glycoprotein composed of two chains (AA or BB) or a combination of the two chains (AB). The five isoforms are known to activate cellular responses through two different receptors (PDGFR-α, PDGFR-β). In canine derived flexor tendon cells PDGF-BB increased cell proliferation and collagen production *in vitro* (106). In contrast, no changes in cell proliferation could be observed in equine flexor tendon-derived cells treated with PDGF-BB, although increases in type I collagen production were demonstrated (123). Gene transfer of *PDGF* had a more pronounced influence on type I collagen production as compared to gene transfer of *VEGF* in rat tenocytes *in vitro* (124). The neutropins include the nerve growth factor (NGF) and the brain-derived neurotropic factor. Their receptors (TrkA, TrkB, p75) have been identified in tenocytes derived from human Achilles tendon (125). Berglund et al. were able to show that NGF was present in rabbit flexor tendon and sheath, but its expression was not increased during flexor tendon repair (126).

4.7 Treatment of tendon injury in the horse

The ultimate goal of any treatment and management program is to maximize the chances for a tendon to repair with adequate strength and elasticity for a return to a similar level of performance, along with the lowest risk for re-injury within an appropriate period of time (127).

It was in 1964 that Asheim described the common treatment of tendon and ligament injuries (128). These basic principles of cooling, support and rest remain integral parts of what is generally accepted and still widely used as the conventional treatment for tendonitis in the horse in many equine hospitals. Cold therapy in the inflammatory phase acts anti-inflammatory and analgetic largely through vasoconstriction, decreased enzymatic activity, reduced formation of inflammatory mediators and nerve conduction. A supportive bandage reduces inflammation and edema by increasing interstitial hydrostatic pressure. In more severe cases with complete disruption of a flexor tendon, the supportive bandage can be complemented with a splint or cast to support the metacarpo-/metatarso-phalangeal- or the coffin joint. For further reduction of the overwhelming inflammatory phase as well as for analgesia and thereby the well-being of the horse, non-steroidal anti-inflammatory drugs are used. Systemic steroids are thought to be more anti-inflammatory than non-steroidal anti-inflammatories but inhibit fibroplasia and hence repair of the tendon. A further risk of systemic steroids in the horse is the induction of laminitis. For local anti-inflammatory action 20% medical grade dimethyl sulfoxide (DMSO) is used topically as a dressing of the bandage. What was formulated as rest for the horse represents an exercise rehabilitation program based on the severity of the initial injury and the progressing ultrasonographic appearance of the lesion. Depending on the initial trauma to the tendon, the rehabilitation time period varies from between 6 and 12 month, and even longer in very severe cases. At the basic level, conventional therapy aims to alleviate the overwhelming inflammation, prevent adhesion formation and exert a positive influence on scar tissue remodeling. It has been reported that 23-67% of horses with tendon injury treated using conservative methods will re-injure their tendons within 2 years of the original injury (129, 130).

The extended period of convalescence, the limited quality of the repair tissue and the high risk of relapse, have stimulated interest in the development of new strategies for the treatment of tendonitis in the horse. So far, most treatment regimens have failed to prove efficacy or were at least not superior to conventional treatment. Intralesional therapies, including polysulfated glycosaminoglycans (PSGAGs) (131, 132), hyaluronic acid (HA) (133, 134) and beta-aminopropionnitrile fumarate (BAPN) (135) have been investigated as alternative treatments to improve tendon healing. PSGAGs inhibit collagenase, MMPs and the activation of macrophages (43),

but have shown no effects on proteoglycan production by fibroblasts (132). However, although one study reported a success rate of 76% for return to athletic function for horses treated with PSGAGs compared to 46% for control horses (129), no difference between treated and control horse could be found in another study (130). The use of intralesional HA failed to improve outcome when compared to control animals (130). In contrast the use of intrathecal HA decreased the inflammatory cell infiltrate, intralesional hemorrhage and intrathecal adhesion formation (133, 136). BAPN inhibits the lysyl oxidase enzyme which is responsible for the formation of collagen crosslinks and thereby would allow for more normal parallel fiber arrangement. With the use of BAPN, a normal ultrasonographic appearance of clinically injured tendons was seen at 20 weeks post treatment (137). An *in vitro* study revealed reduced collagen synthesis of equine tendon-derived fibroblasts under the influence of BAPN. The authors therefore concluded that BAPN might delay tendon healing (135). Yamamoto et al. compared intralesional BAPN and HA in a collagenase-induced injury model in rabbit Achilles tendon. Whereas a positive effect of HA on fiber diameter was detected, this was not the case for the BAPN treated group (138). One clinical study reported a decrease in the re-injury rate for the affected limb being treated with intralesional BAPN (16% re-injury rate) compared to intralesional PSGAGs or HA and systemic PSGAGs (42.5 to 44.4%) over a 2 year observation period (130). Extracorporeal shock wave therapy has demonstrated significant improvement in prognosis over conservative treatment for chronic hind limb proximal suspensory ligament desmitis (139, 140), most likely through induction of analgesia (141). However, besides studies in laboratory animals, where histological differences in granulation tissue where found (142), no scientific evidence for its application in the assistance of equine flexor tendon healing have been reported. Surgical treatment strategies are equally controversial. Percutaneous tendon splitting was initially advocated to treat chronic cases of tendonitis as early as 1931. Later, investigators found that tendon splitting resulted in increased trauma and granulation tissue production, no alterations in collagen production and continued lameness (143, 144). More recently, tendon splitting has been revisited as an effective technique for the management of acute, rather than chronic tendonitis, where it was thought to allow evacuation of the intratendinous hematoma and edema, reduce lesion size and improve collagen fibril orientation (145). To date, controversy exists between investigators as to the long-term effectiveness of tendon splitting because the quality of repair and return to race form are variable and often not superior to other treatment methods (145). Desmotomy of the accessory ligament of the SDFT (ALSDFT) to treat SDFT tendonitis is also considered controversial. The rationale behind this desmotomy is to increase the involvement of the SDFT muscle and thereby reduce the peak loads on the SDFT at full weight bearing in the remodeling phase of the healing process when

the animal returns to work (146). A later *in vitro* investigation into the biomechanical effects of desmotomy of the ALSDFT determined that the strain and metacarpophalangeal joint hyperextension increased following transection of the ALSDFT, which would appear to be counterproductive (147). A study reported by Gibson at al. found that horses after desmotomy of the ALSDFT were more than 1.3 times more likely to race on 5 or more occasions, but were also more than 5.5 times more likely to experience SL injury (148).

The poor clinical outcome associated with tendon injury and the limited capacity for regeneration of injured tendon have resulted in a growing interest in the use of tissue engineering approaches for tendon therapy in both man and animals (149). Furthermore, biologically based strategies aimed at increasing cell activity and production of extracellular tendon matrix proteins are growing in popularity (150, 151).

4.7.1 Growth factors and cytokines for tendon healing

Different growth factors have been observed in tendon and during tendon healing, and have been, at least in part, evaluated for their potential to improve tendon healing. IGF-1 is a mitogen that stimulates ECM synthesis. Additional benefits are an increased DNA and collagen synthesis, improved echodensity of tendon and a trend towards increased stiffness (110). Currently, no clinical studies exist in which the re-injury rate after IGF-1 treatment has been examined. Therefore, further studies are needed before the use of IGF-1 can be recommended for clinical cases of tendon injury. TGF-β has also been considered for intralesional treatment, but clinical evidence is lacking. In a prospective 3-year study, 10 horses with SDFT tendonitis and 1 horse with SL desmitis were treated with TGF-β1. Up to 60% of horses had palpably enlarged tendons in the treatment area. All horses returned to their previous level of athletic use, but 40% injured the contralateral, untreated limb. This led to an overall re-injury rate comparable to conservative treatment, even though it is known that many, if not all, strain induced tendon injuries in race horses have bilateral components (30, 152). Besides the use of single growth factors, mixtures of growth factors are under mostly clinical evaluation. Treatment with intralesional bone marrow aspirate has been evaluated for the treatment of SL desmitis, where a success rate of 84%, with these horses returning to full work has been reported (153). Bone marrow is a rich source of growth factors (154) but contains very few MSCs (1 in 10^4 nucleated cells). Hence this treatment more closely resembles a growth factor treatment. Over the past decade, Platelet rich plasma (PRP) has gained increasing attention in human orthopedic sports medicine as well as in the treatment of equine tendon injury. PRP can be created using centrifugation

or through gravity filtration of autologous blood. The α-granules of the platelets contain, among others, PDGF, TGF-β, VEGF, IGF-1 and EGF, which are released on platelet activation. The definition for PRP in the literature is somewhat variable. In literature concerning human medicine, any autologous plasma with a platelet concentration above baseline blood values (155) or a concentration of at least two times (156) to more than five times (157) above baseline values is defined as PRP. In equine medicine PRP is generally accepted to have a platelet concentration of at least two to four times above plasma level. Preparations with lower platelet concentrations are therefore termed autologous conditioned plasma (ACP). A novel approach to better characterize PRP preparations and to allow for better comparison of published results is by using the PAW classification system, taking the absolute numbers of platelets, the manner in which platelet activation occurs and taking the presence and/or absence of white blood cells into account (158). Beside its use in oral and maxillofacial surgery to accelerate peri-implant and soft tissue healing (159-163), PRP has also been investigated for regeneration of bone (164, 165), cartilage (166) and ligament (154). PRP has been demonstrated to enhance the expression of *COL1A*, *COL3A* and *COMP* in equine tendon explants, with no associated increases in *MMP3* or *MMP13* (167). In a surgically induced model of equine tendinopathy, the intralesional administration of PRP resulted in modulation of composition, organization and biomechanical properties (168). Also, reports on small case series suggest efficacy of intralesional PRP (169) although long term clinical data on a sufficient number of cases have yet to be published.

The acellular matrix of porcine urinary bladder submucosa (ACell™) is marked as an additional therapy for tendon injury. It has been proposed that ACell™ delivers extracellular matrix components and growth factors to the damaged region of the tendon and may attract MSCs to the site through the release of bioactive breakdown products (170). Beside anecdotal reports that this treatment improves tendon healing, one study investigating ACell™ usage following collagenase induced tendinitis failed to prove any positive effects of this treatment (171). Autologous conditioned serum (ACS), marked in veterinary medicine under the trade name IRAP™, was initially introduced on the marked to treat equine osteoarthritis. Through a conditioning process, autologous leukocytes in the presence of chromium sulfate coated medical grade glass beads produce the anti-inflammatory mediator interleukin-1 receptor antagonist (IL-1Ra). An increase of a 140-fold of the concentration of IL-1Ra through the conditioning process was proven in human subjects (90). After sterile filtration, the ACS is then injected into the affected joint, were the competitive receptor antagonist is thought to block the inflammatory cascade. Besides the increase in IL-1Ra through the conditioning process, levels of FGF2 and TGF-β1 were increased up to 750% and 182% in human subjects (87). It is the

increase in growth factors that has encouraged the use of ACS to treat soft tissue injuries. Treatment of muscle injuries in both mice and humans by local administration of ACS resulted in accelerated muscle regeneration (87,89). Dahlgren et al. evaluated the effect of ACS on normal tendon explants. They found no significant difference in GAG, COMP or type I collagen between normal equine serum and ACS treated cells (172). Easter and Watts presented a case series of 272 horses treated with ACS for proximal suspensory desmitis at the 60th Convention of the American Association of Equine Practitioners in Salt Lake City, Utah in 2014. They reported a high success rate combined with a shortened convalescence period, but included just cases with no or only minor fiber disruption, and provided no control group (173).

Experimental evidence has shown that fetal tissue in the early mid-gestational phase responds to injury in a fundamentally different manner than does adult tissue (174). In general, fetal wound healing occurs at a faster rate and in the absence of scar formation. In addition to skin, fetal articular cartilage, nerve and bone have demonstrated the ability to heal without scarring in experimental models (175-181). Flanagan et al. found evidence for scar-less healing also in fetal sheep tendon (182). It is widely believed that the differential expression of growth factors and cytokines in fetal tissue in the early gestation period compared with that in the late gestational period and in adult tissue plays an important role in this distinct healing process (174, 175, 183). Interestingly, mechanical and chemical factors used in many tissue engineering strategies are those identified in adult tendon homeostasis and healing, even though these factors direct adult tendon healing towards scar formation rather than tissue regeneration (184). A new paradigm is to use embryonic developmental factors as cues to promote tendon regeneration, although the majority of soluble factors regulating embryonic tendon development are unknown (31). It is thought that TGF-β signaling plays a central role in the induction of skeletal progenitors toward the tendon fate (185).

4.7.2 Cell-based tendon therapy

The prospect of regenerating damaged or diseased tissues has led to a vast variety of cell-based therapeutic strategies. A sub- or side-population of precursor cells (tissue-specific progenitor cells) that are used to replenish cells lost because of natural turnover and to aid in post injury repair has been identified in most tissues (149) and may represent an interesting cell source. Notable examples are the muscle satellite cells (186-189), multipotent cells from the brain (187), cartilage (189, 190), trabecular bone (191), placenta (192), liver, lung, spleen, thymus and pancreas (189). Multipotency has also been shown for cells derived from tendon (193), but the exact side for these cells within the

tendon is not known. Fibroblasts derived from tendon or other sources are under investigation as well (194). However, the potential formation of a secondary lesion in the tendon at the donor side, along with differences in cell characteristics that arise in different tendons and regions of tendon (195) may not be ideal. Dermal fibroblasts, which were shown to be capable of functionally bridging a tendon defect and to have similar histological and tensile properties to a tenocyte seeded scaffold (196), behaved differently from tenocytes *in vitro* (197).

Stem cells have two basic characteristics; they are able to continually self-renew and are capable of differentiation into multiple specialized cell types (198). Furthermore stem cells have been shown to produce several growth factors, interleukins and other cytokines (199). Different stem cell sources have been considered in the horse. Embryonic stem cells (ESCs), even though exhibiting real pluripotency, carry the disadvantage of being allogenic and being associated with the risk of teratoma formation (200-205), although teratoma formation has so far not been shown for equine ESCs. The risk for teratoma formation may be even higher in induced pluripotent stem cells (iPSCs) (206).

Within the equine orthopedic field, MSCs have been used experimentally and in limited numbers in clinical cases to treat subchondral cystic lesions, for bone-fracture repair (207) and cartilage repair (208, 209). However, the most common clinical application of MSCs is in the treatment of overstrain-induced tendon injuries. The predominant cell type currently in use are autologous MSCs, which can be harvested in sufficient numbers from bone marrow (sternum, tuber coxae), adipose tissue and the umbilical cord (210, 211).

Critical to tendon tissue engineering is the induction and guidance of stem cells towards the tendon phenotype. Several key factors can influence the differentiation of MSCs. Of those, the mechanical environment, contact with resident cells and the extracellular matrix, as well as soluble growth factors, are of special importance. The paracrine signals that fine-tune MSCs to the unique tenocyte fate during normal development are virtually unknown, but they appear to involve FGFs (212) and key members of the TGF-β family, such as GDF5, 6 and 7 (213). Several studies have explored the use of various growth factors promoting tenogenesis (95, 121, 214, 215). Just recently TGF-β2 was identified as a potent tenogenic factor for tendon progenitor cells and MSCs (95). Hoffmann et al. demonstrated the expression of tenogenic markers *in vitro* and tendon tissue formation *in vivo* with transfected MSCs expressing *SMAD8* and *BMP2* (216). In line with the mechano-active nature of tendon, it seems that mechanical forces play a role in promoting tenogenesis. A number of recent studies have emphasized the importance of mechanical regulation of tenogenesis using MSCs (217-221). Other studies have demonstrated positive effects of mechanical stimulation on cell

proliferation, type I collagen production and cell alignment of MSCs (222). In comparison to adipogenic, osteogenic and chondrogenic differentiation, little is currently known about the precise signaling pathways and molecular mechanisms responsible for the differentiation of MSCs into tendon fibroblasts (223). The current lack of a phenotypic marker for tenocytes *in vitro* makes it difficult to establish a culture system for their generation from stem cells (224) and to determine whether MSCs have the ability to differentiate into tendon fibroblasts (225).

There are a number of experimental animal models, which have demonstrated efficacy of MSC implantation over controls, usually using laceration models in rabbits or rats (226-228). Characteristics of overstrain tendon injuries in the horse lend themselves for a tissue engineering approach based on the fact that lesions are usually core lesions, providing a natural enclosure for implantation and are filled with granulation tissue by the time of implantation, which acts as a scaffold. Furthermore, neovascularization of the defect area provides nutritional support for the implanted cells. Through the intratendinous location of the defect, anabolic factors and mechanical stimuli are provided.

Promising preliminary results were demonstrated following the treatment of tendonitis of the SDFT with bone marrow-derived MSCs (BMSCs), where an 18% decrease in the re-injury rate was observed as compared to 56% after conventional treatment (229, 230). A shorter post treatment observation period (> 1 year for the BMSC group as compared to >2 years for the conventional treatment group) is a limiting factor in this study.

Despite stem cell therapy having proven high potential with no known side effects, the mode of action of the implanted cells remains unknown. One of the basic characteristics of stem cells is their capability to differentiate into multiple specialized cell types. Furthermore, stem cells have been shown to produce several growth factors, interleukins and other cytokines (199). Because of this, three distinct mechanisms for their mode of action in tissue regeneration are feasible, (i) the cells secrete factors which stimulate the regenerative capacity of the tendon such as VEGF, bFGF or IGF; (ii) the cells become integrated into the damaged tendon and differentiate into tenocytes and (iii) a combination of (i) and (ii). Whereas the amount of cells is crucial for a reliable paracrine effect, cell retention is key for functional integration of the transplanted cells. Therefore, the application format will play an essential role in the development of efficient cell-based therapies to regenerate the tendon.

Cell-based therapy to regenerate diseased tendon tissue has shown high potential and has attracted considerable interest in both the scientific and clinical communities. As for many of the therapeutics presently available, technology and marketing are ahead of laboratory and clinical research.

Current techniques used in the clinics are simple and likely to require more sophisticated methodologies to optimize efficacy. To optimize the therapeutic concept, unanswered questions like the differentiation capacity of the applied cells, the mode of action and most suitable application format, need to be answered by further scientific evaluation. Future research will unveil mandatory data about cell-based therapy and thereby improve treatment strategies in the horse, other animal species and humans.

4.8 Experimental models of tendon injury

A valid model of tendinopathy/tendon injury should replicate the clinical, histopathological and functional characteristics seen in the naturally occurring injury. However, no single animal model can fully reproduce the naturally occurring situation. Therefore, several animal models may have to be used in a concerted effort to study the different aspects of tendon injury and healing.

Naturally occurring tendinopathy/tendon injury is common in racing dogs and in horses. Even though there are obvious anatomical differences between quadrupeds and bipeds, individual animal species have naturally occurring strain-induced injuries of specific tendons with similarities to specific human tendinopathies. The superficial digital flexor tendon of the horse for example, shows many similarities to the human Achilles tendon. As naturally occurring tendon injuries varies significantly and are impossible to control, they are not suitable for controlled studies. On the other hand, future success in their treatment may stimulate management of tendinopathy in humans.

Induced tendinopathy models fall into two categories. Mechanical loading is the most frequently reported intrinsic factor for tendinopathy and therefore many current animal models have been developed based on mechanical overloading of tendon. A second category of model involves the introduction of chemicals reported in tendinopathy samples, into normal animal tendon. The most commonly used species for induced tendinopathy are rats and rabbits. They are less costly, easily available and more biologic tools are available as compared to larger animals. Three different methods for mechanical overloading are used. In forced treadmill running, the animal is forced to run on a treadmill to mimic tendon overuse. This method has been used to develop tendinopathy in the supraspinatus tendon and Achilles tendon of rats. Increased cellularity, glycosaminoglycan content, collagen disorganization, changes in cell morphology, larger cross-section area, a decrease in elastic modulus and maximum stress have been observed (231-237).

Artificial muscle stimulation has been used to induce tendinopathy in the flexor digitorum profundus of rabbits and the Achilles tendon of rabbits and rats by electrical stimulation of muscle via surface electrodes with the animal under general anesthesia (238, 239). An increase in tear size, tear area and tear density have been reported. However, no tenderness, lameness, swelling or reduction in the range of motion were noted (238).

In direct repetitive tendon stretching, direct tendon overloading is induced by stretching the tendon repeatedly and directly with a single application of sub-failure cyclic load using an external device (240, 241). Inter-fiber space widening, matrix disruption, fiber angulation, thinning and discontinuities were accompanied with a marked increase in the mRNA expression of type I, II and V

collagen and with an increase in *MMP13* and *IL-1β* expression. However, histology showed no inflammation up to 3 days post stretching.

One of the limitations of the artificial muscle stimulation and the direct repetitive tendon stretching method is the single intervention to induce tendon damage, which therefore does not mimic the more gradual course of development of overuse-induced tendinopathy.

The intratendinous injection of chemicals is used to establish animal models of tendinopathy, using chemicals, which have been observed in clinical samples of tendinopathy. Chemicals which have been used to induce tendon injury animal models include collagenase, cytokines, PGE_1, PGE_2 and fluoroquinolone.

An intratendinous injection of collagenase has been widely used to produce lesions in the SDFT, the DDFT, the Achilles tendon, patellar tendon and the supraspinatus tendon in horses, rabbits and rats (110, 242-246). In rabbit patellar tendon the injection of collagenase increased angiogenesis, hyper cellularity, focal fibrosis and collagen-bundle disarray. The cross sectional area of the tendon was increased and collagen content significantly decreased at 4 weeks (243). In the horse, core lesions within the SDFT were induced with collagenase and were characterized by extracellular matrix damage, loss of spindle-shaped fibroblasts, increased expression of type III collagen and decreased mechanical properties (110, 245).

Stone at al. (243) injected cytokines and cell-activating factor (CAF) into rabbit patellar tendon. There was no change in tendon cross-sectional area and the matrix appeared intact. Only a transient increase in cellularity was observed, along with a significant decrease in the ultimate load at 16 weeks. As the cytokine and CAF mixture is not present in naturally occurring tendon injury, the validity of this model is questionable.

The intratendinous injection of prostaglandins to induce tendon injury is based on the increased expression of prostaglandin E_2 (PGE_2) and cyclooxygenase (COX) -2 in clinical samples of tendon injury, the increased production of PGE_2 during exercise and the increased production of COX-1, -2 and PGE_2 in human tendon fibroblasts after repetitive mechanical loading *in vitro* (64, 247-249). Repeated injections of PGE_2 in rabbit patellar tendon led to focal hyper cellularity and matrix degeneration (250). Sullo et al. reported an inflammatory response followed by degenerative changes including increased vascularity, cellularity and fiber disorganization after PGE_1 injection into the peritenon (251). As PGE_1 has not been demonstrated in naturally occurring tendinopathy, the reliability of this model is uncertain. Fluoroquinolone antibiotics have been implicated in the etiology of tendinopathy in humans. Especially the Achilles tendon, but also the biceps brachii, the supraspinatus and extensor pollics longus tendon have been affected (252). In fluoroquinolone-

associated tendinopathy, hypercellularity, increased GAG content and mucoid degeneration was detected. Furthermore, increases in tendon thickness and hypoechogenic areas in ultrasonographic imaging were noted (253, 254). In dogs treated orally with ciprofloxacin for 5 days, the amount of type I collagen, elastin, fibronectin and β1-integrin was decreased in the Achilles tendon (255). As fluoroquinolones are not involved in the majority of tendinopathy cases, their use to induce a model of tendinopathy remains questionable.

The collagenase induced tendon injury model is the most commonly used model in the horse. A major limitation of this model is due to the strong inflammatory response that extends the peritendinous tissue, either due to leakage of the collagenase through the injection site or because it causes a melting tendonitis that erodes from the center of the tendon outwards, and thus destroys the superficial tendon layers and the paratenon. Because of this, the precise location, size, shape and volume of the core lesion are difficult to control. Surgical models of tendon injury have been explored sporadically as alternatives to enzymatically induced tendon injuries in horses (256-258), as well as in rabbits, sheep and dogs (259, 260). Such models relied on either surgical removal of a segment of the tendon, by incising the paratenon and the outer surface of the tendon, or on complete transection of the tendon. Quite recently, a new model of tendon injury within the SDFT of horses has been established (261), which is thought to overcome the limitations of the collagenase-induced injury, as well as other existing surgical models. Under general anesthesia and ultrasonographic guidance, a synovial resector is advanced longitudinally in the metacarpal region of the SDFT creating a core lesion. The surgically created core lesion is further advanced by a postoperative exercise program. Even though this model does not mimic every aspect of naturally occurring tendon injury, it has proven to be suitable for evaluating the effect of intralesional injection of PRP to stimulate tendon healing (168).

The use of animals for experimentation raises many ethical concerns about the animal welfare. To allow for informed scientific and animal welfare decisions, experiments must have the clear objective of improving the welfare of man and/or animals, and the researchers need to constantly keep animal welfare at the forefront to ensure humane treatment of all animals. Following the principles of the three Rs (replacement, reduction and refinement) many basic research questions may be possible to answer with *in vitro* experiments, especially as new culture formats become available, more closely resembling the *in vivo* environment and therefore generating more reliable data.

4.9 *In vitro* cell culture of tendon-derived fibroblasts

Cell culture systems provide many advantages to address different research questions. They not only enable monitoring of the exact response of cells to different treatments, but also allow for the characterization of a specific cell type. The expansion of differentiated tenocytes *in vitro* is a useful approach to analyze tendon healing and to develop tools to support tendon repair with the use of tendon tissue engineering strategies (262). Tendon-derived fibroblasts can be isolated from native tissue by explant culture (167, 172, 262-264) or by collagenase digestion (265-271). A more recent study revealed a higher cell yield beside a faster time interval for cell isolation using enzymatic digestion compared to cell migration from explant culture. Interestingly, type I collagen expression was higher in cell cultures using enzymatic digestion, whereas decorin expression was higher in cell cultures generated from cell migration (272).Tendon-derived fibroblasts in monolayer culture typically exhibit a spindle shape, fibroblast-like appearance, making it impossible to differentiate tendon-derived cells from other fibroblastic cells. Therefore characterization of tendon-derived fibroblasts *in vitro* is carried out by gene expression analysis and identification of ECM proteins, like decorin and COMP.

4.9.1 Gene expression markers of tendon-derived fibroblasts

There is a paucity of data regarding molecular markers that identify the phenotype of the tendon cell. Type I collagen (*COL1A2*), the major component of the extracellular matrix of tendon is expressed in a large variety of tissues and cell types and is therefore not specific to tenocytes. The same is true for type III collagen (*COL3A1*), which is known to be upregulated during tendon healing, but again is not specific to tendon tissue. Even though type II collagen (*COL2A1*) is present in tendons (265), especially in locations of where compressive forces act, it is recognized, together with type X collagen (*COL10A1*) and the transcription factor SOX9 as being representative for chondrogenic differentiation (190). The transcription factors scleraxis (SCX), Six2, Fibin, Mohawk, Erg1 and Erg2 have been identified in tendon progenitors (273-277). Of these, the basic helix-loop-helix transcription factor scleraxis has been highlighted as a specific marker of tendon progenitors (278-281). Scleraxis plays essential roles in mesoderm formation (282) and marks the tendon progenitor population that forms the fourth somatic compartment the “syndetome”, and is continuously expressed through differentiation into the mature tenocyte and ligament cells (279, 283). The classic ERK MAP kinase pathway has been identified as playing an important role in the regulation of *SCX*

gene expression by FGF signaling (284). *SCX* null mutant mice have been shown to have distinct tendon defects and tenogenic differentiation is inhibited (278).

Besides being discussed as a marker for tendon progenitors, scleraxis has been proposed as a tendon specific marker by several authors (275, 279, 285). Lejard et al. were able to show that scleraxis activates *COL1A1* gene expression in tendon fibroblasts (223). Espira et al. and Maeda et al. could show within rat cardiac fibroblasts, myofibroblasts and in Achilles tendon-derived fibroblasts from transgenic mice, that *SCX* gene expression was dependent on TGF-β signaling and that upregulation of *SCX* led to increased *COL1A2* gene expression (214, 286). Furthermore scleraxis regulates the expression of tenomodulin (*TNMD*), another potentially tendon specific maker (267). As *SCX* is expressed in other skeletal lineage cells such as muscle, cartilage and bone, some authors question its use as a tendon specific marker (224, 225).

Tenomodulin has been reported to represent a good phenotype marker for tendon fibroblasts (267, 287) and is claimed to be a selective tendon gene (225). Mice lacking tenomodulin have tendons with a disrupted fibril structure and exhibit severely reduced tenocyte proliferation (288). The soluble form of tenomodulin, containing the C-terminal ChM-I like domain, inhibits the proliferation, migration and tube formation of vascular endothelial cells (289). Tenomodulin is a type II transmembranous glycoprotein that is preferentially expressed in dense connective tissue, including tendons, ligaments and the eye (cornea and sclera), but low levels of mRNA transcripts have also been identified in muscle, thymus, hearts, liver, spleen, nervous tissue, lungs, cartilage and dermis (287, 290-295). The high expression of tenomodulin compared to different other tissues is fading when cells are cultured in 2-D systems (69), questioning its use as a tendon specific marker. Irrespective of this, Oshima et al. claim that the combination of scleraxis as an early marker and tenomodulin as a late marker should enable clarification of the molecular mechanisms underlying tenocyte differentiation and tendon regeneration (289).

Tenascin has been employed as a tendon marker, although it is also expressed in cartilage and nerve (224, 294, 296). Tenascin-C (TNC) is an anti-adhesive ECM protein and present in musculoskeletal regions that are transmitting mechanical forces from one tissue component to another (297). Tenascin-C contains a number of repeating fibronectin-III domains, and following stress induced unfolding of these domains, it also functions as an elastic protein (298, 299). The expression of *TNC* is regulated by mechanical stress (300).Tendon regions with a fibrocartilagenous phenotype that are subjected to compressive loads have been shown to have higher levels of *TNC* (295, 301, 302). Isoforms of tenascin-C in degenerated tendon were suggested to potentially stimulate tenocyte proliferation (303). Tenascin-C may also play a role in collagen fiber alignment and orientation (304).

As many of the key matrix genes are expressed in a variety of mesenchymal tissues and therefore are not sufficiently discriminatory (265), there are currently no absolute specific molecular markers that can be used to characterize tenocytes (305). Some authors therefore recommend the use of a panel of marker genes, such as *COL1*, *SCX* and *TNC*, to identify the tendon cell phenotype from other mesenchymal tissues (265). Besides the use of more or less specific marker genes, histological analyses and mechanical testing are used to verify the success of tendon tissue engineering interventions (306).

4.9.2 Phenotypic drift

Cell dedifferentiation is a common biological process in cultured cells deprived of their *in vivo* niche. For example, bovine articular chondrocytes become dedifferentiated after being cultured in monolayer for three weeks and lose expression of type II collagen (307). The same has been shown for rabbit articular chondrocytes (308) and chick chondrocytes (309). Already in 1976 Schwarz et al. noted, that the loss of differentiated function occurred during prolonged monolayer culture of avian tenocytes (310). Extended monolayer culture of tenocytes has been reported to lead to decreased levels of type I collagen and decorin with continued passages (311). Other authors have reported low expression levels of *TNMD*, observed as an initial reduction and ultimately leading to a total absence over the course of extended culturing (265, 266, 269, 294). Jelinsky et al. described a rapid down regulation of tendon selective genes (*TNMD* and thrombospondin4 (*THBS4*)) in rat primary tendon surface cells and internal fibroblasts grown in 2-D cell cultures suggesting that tendon cells in culture rapidly dedifferentiate (225). Studies on avian and rabbit tenocytes have indicated that phenotypic drift may manifest at early passages (310, 311). Yao et al. reported a decrease in confluent culture cell density, a decline in alamar blue reduction, a change in cell morphology from spindle shaped to more rounded, an increase in the ratio of type III to type I collagen and a significant decrease in decorin apparent by passage 4. It was therefore concluded that tenocytes in monolayer culture display an unstable phenotype and tend to dedifferentiate (312). Additionally Stoll et al., evaluating human primary tenocytes isolated from hamstring tendons in 2-D and 3-D cultures, observed a significant decline in decorin (*DCN*), aggrecan (*ACAN*) and *SCX* gene expression as well as a decrease in *COMP* in the 2-D cultures. It was suggested that these tendon ECM proteins might therefore act as predictive indicators of tenocyte dedifferentiation (262). A lack of appropriate cell-matrix interaction (313), cell-to-cell interaction (263, 314) mechanical stimulation (69) are discussed as possible cues for tendon cell dedifferentiation.

In order to allow for the expansion of cell numbers for tissue engineering approaches as well the development of *in vitro* models to evaluate different treatment modalities, phenotypically and functionally stable tenocytes, which maintain a differentiated state, are a mandatory prerequisite. As connective tissues are known to have unstable phenotypes and retain the ability to return to an earlier stage of differentiation or to transdifferentiate along other cell lineages (193, 315, 316), cell culture conditions need to be optimized in order to maintain a tendon phenotype (225) or to allow for re-differentiation.

Maintaining tendon-derived fibroblasts in a differentiated state or to re-differentiate tendon cells *in vitro* has been attempted through the use of different culture formats, the addition of different growth factors or through mechanical stimulation.

4.9.3 3-D cell culture

Ideally, cell culture models should reflect the characteristics of the tissue from which they were derived, even after being grown in culture for long periods of time (317). Evans and Trail (197) observed that tenocytes naturally form 3-D structures during *in vitro* culture. Recent investigations have highlighted significant differences in cellular gene expression in monolayer compared with 3-D cultures (268). The 3-D cell culture methods are thought to more closely mimic the *in vivo* cellular environment (268, 318) and represent a possible means by which to stabilize the tenocyte phenotype, thereby providing more intimate cell-cell and cell-matrix contacts compared with monolayer cultured tenocytes (263). The ECM transduces physiological signals to the cells through integrins, which not only regulate cell growth and proliferation but also cell differentiation and matrix remodeling (319). Sawaguchi et al. were able to show that the switch from monolayer culture to 3-D culture led to an up regulation in certain cell-surface proteoglycans, such as CD44, syndecan-2 and syndecan-4, which can modulate the response of cells to pericellular matrix molecules and soluble stimuli such as growth factors and cytokines (268). In the study by Zhu et al., the authors noted a change in cell morphology of tendon-derived fibroblasts in monolayer culture from elongated to a more spread out morphology, which was accompanied by a reduction and finally loss of *TNMD* expression. Culturing these dedifferentiated cells on micro-grooved silicon membranes restored the elongated morphology and *TNMD* expression (269). Therefore cell shape appears to influence, and also reflect the state of differentiation. Cell culture formats should aim to preserve or restore the phenotype of tenocytes observed *in vivo*.

High-density 3-D cell cultures devoid of any scaffold material have been utilized for the culture of tendon-derived fibroblasts. Schulze-Tanzil et al. cultivated human tenocytes in high-density cultures and detected a decrease in type I collagen as well as an increase in *SCX* expression over time. To rule out transdifferentiation of cells to fibrochondrocytes, type II collagen was also measured. However, expression levels of type II collagen were found to be comparable to monolayer cultures. Therefore, they concluded that this culture format seems to promote differentiation of tenocytes (263). Unfortunately, *TNMD* expression was not investigated.

Stoll et al. compared high-density 3-D tenocyte cultures and tenocytes cultured on polylactic-coglycolic acid- (PLGA) scaffolds to normal tendon tissue. In 3-D culture, a morphological shift from a tenoblastic cell shape with round nucleus, towards a more elongated tenocyte phenotype was observed. Type I collagen expression was increased in all culture formats compared to normal tendon, with a decrease over time in 3-D culture. Type III collagen expression was significantly increased in high-density 3-D cultures. Decorin expression was higher in 3-D cultures as compared to 2-D cultures, and *COMP* expression was only increased in high density 3-D cultures. *SCX* expression levels were reduced in all culture formats as compared to normal tendon, but were elevated in monolayer-expanded cells transferred to high-density 3-D cultures. The increase in *SOX9* was not significant in any culture format. However, no changes in type II collagen protein could be detected. β1-intergrin cell matrix receptor gene expression remained mainly unaltered in all culture conditions. Based on these results, the authors concluded that high-density 3-D culture as well as PLGA-scaffolds might promote tenocyte re-differentiation (262).

Schneider et al. evaluated the influence of various different culture conditions on the promotion of MSC tenogenesis, including different growth factors, the conditioned medium from tenocyte cultures and co-culture with primary canine tenocytes. High-density 3-D cultures of tenocytes alone served as controls. Although *SCX* and *TNMD* expression could be induced in high-density 3-D cultures of MSCs under all conditions tested both genes were also expressed in unstimulated controls (264).

Taylor et al. evaluated gene expression markers of equine tenocytes in tissue samples as well as in 2-D and 3-D culture formats. Type I collagen expression levels were higher in the 3-D cultures as compared to monolayer cultures. *SCX* expression levels were comparable in both culture systems, but remained reduced as compared to normal tendon. *TNMD* gene expression was not detected in either *in vitro* culture system (265). Because of the short cultivation period of the tendon cells in the collagen gel, conclusions should be drawn with caution.

Another interesting 3-D cell culture format is the gravity-assisted formation of microtissues (MTs) through the cultivation of cells using the hanging drop system. MTs combine advantages of

engineered tissues, such as the 3-D environment to maintain a differentiated state, and an accelerated extracellular matrix production. Applying this technology, the generation of artificial MTs from different cell types has been made possible and has included primary human chondrocytes and rat hepatocytes, primary human fibroblasts, embryonic sensory neurons, and neonatal rat and mouse cardiomyocytes. Even cultures of two or more different cell types displayed cellular reorganization into a more *in vivo*-like morphology (320-324). Mirsaidi et al. cultivated adipose-derived stromal cells (ASCs) in the MT format (ASC-MT). Besides demonstrating osteogenic differentiation *in vitro*, injection of predifferentiated ASC-MTs into the tibia of an osteoporotic mouse model significantly improved trabecular bone quality (325, 326). Up until now, the MT format has not been evaluated for the use of culturing tendon-derived fibroblasts.

4.9.4 Growth factors

Embryonic tendon progenitor cell differentiation *in vivo* is regulated by a combination of mechanical and chemical factors (185). Although the mechanisms responsible for the regulation of embryonic tendon development remain largely unknown (31), the role of certain growth factors has been, at least in part, elucidated. FGF has been identified as playing an important role in the induction of tendon progenitors (215) and has been proposed as a likely means through which muscles influence tendon formation and survival (327). FGF4 is expressed by myotubes in zones close to the tendons, and local application of FGFs in normal limbs upregulates the expression of the tendon marker *SCX* and promotes proliferation of *SCX*-positive cell progenitors (285). Furthermore, FGFs induce the expression of *Erg1* and *Erg2*, which are transcription factors expressed in tendons responsible for the maturation of the tendon tissue (275). FGF and TGF-β signaling have been shown to induce early tendon markers (215) and Lorda-Diez et al. demonstrated a central role for TGF-β signaling in the induction of skeletal progenitors toward the tenocytes (185). Additionally, Schweizer et al. and Seo et al. confirmed an essential role for TGF-β2 and TGF-β3 ligands in tendon formation (279, 328).
There is evidence indicating a role for BMPs in tendon development, but the precise function of BMPs in this process is controversial (221). Expression of *BMP2*, *-4* and *-5* has been detected in developing tendon (329). Macias et al. described BMP7 in developing flexor tendons in chicken limb development (330). Regulators of BMP signaling, including Twisted Gastrulation, Dan, Follistatin and noggin, are expressed in early tendon blastomas (331). It has been proposed that the BMP antagonist noggin, produced by digit cartilage, exerts a positive influence on tendon formation by blocking the negative influence of BMP ligands (279). Sclerostin, which may antagonize both BMP and wnt

signaling, is expressed at different stages of tendon differentiation (331). In contrast with these findings, genetic approaches indicate that a subset of BMPs promote tenogenesis. Structural and functional anomalies of the Achilles tendon were reported in mice deficient for GDF5, GDF6 and GDF7 (332-334), which belong to the BMP family. Wolfman et al. showed that ectopic injection of GDF5, GDF6, GDF7 in rat muscle induces tendon-like tissue (213). Violini et al. claim tenogenic differentiation of BMSCs through *in vitro* exposure to BMP12 (GDF7) as confirmed by enhanced *TNMD* expression. Furthermore a morphologic change from a fibroblast-like to a more tenocyte-like phenotype was observed (335). Wang et al. noted the expression of *SCX* in transfected and BMP12 expressing MSCs (336). Stimulation of rat BMSCs with BMP12 was reported to induce increased expression of *TNMD* and *SCX* in both monolayer culture and in collagen scaffolds. Type I collagen and *TNC* expression was also enhanced in cells in 3-D cultures (121). In rat Achilles tendon-derived fibroblasts, GDF5 supplementation led to an upregulation of type I and III collagen, *TNC*, biglycan, versican and *DCN* (337). In rat ASCs GDF5 led to increases in the expression levels of *SCX*, *TNMD* and *TNC* (338, 339). However, BMP7 could not significantly influence *SCX* or *TNMD* expression in rat patella and Achilles tendon cell culture (340).

Caliari et al. evaluated the effect of different growth factors or combinations thereof, on tenocyte chemotaxis, proliferation, metabolic activity and gene expression in equine tendon-derived fibroblasts grown on collagen-glycosaminoglycan scaffolds. bFGF and GDF5 resulted in a significant upregulation of *SCX* and *TNC* expression levels. IGF-1 was capable of significantly enhancing *COL1A2*, *COL3A1* and *DCN* gene expression. Combinations of IGF-1 and GDF5 increased expression levels of *COL1A2*, *SCX* and *COMP* (341). Human tenocyte culture in a serum-free 2-D system for 14 days with IGF-1 expressed increased levels of *DCN* but not *SCX*, *TNMD* or *COL1*. In contrast, TGF-β3 resulted in increased expression levels of *SCX* and *TNMD*, whereas *COL1* expression was most pronounced with the combination of TGF-β3 and IGF-1. Interestingly, no single factor was capable of restoring the phenotype in serum free culture systems (270). The same was true in a study by Schneider et al. in which the tenogenic differentiation of canine MSCs was evaluated in high-density 3-D cultures. The combination of IGF-1 and TGF-β1 was able to maintain a stable phenotype, although neither was effective when used separately. In contrast to the study by Caliari et al. (341) who utilized equine tendon-derived fibroblasts, IGF-1 alone was not able to induce *DCN* expression in MSCs. *SCX*, *TNMD* and *DCN* expression levels were highest in high-density 3-D cultures treated with both IGF-1 and TGF-β1 combined (264).

Barsby et al. evaluated the influence of the three isoforms of TGF-β on equine ESCs and tendon cells. They were able to show that undifferentiated and differentiated ESCs as well as adult tenocytes *in*

vitro express receptors for TGF-β. Furthermore, of the three isoforms, TGF-β3 had the greatest effect in terms of upregulating *SCX* expression in ESCs. Exposure of ESCs to TGF-β3 increased the expression of type I collagen, *TNC* and *COMP* and by day 7 *TNMD*. Immunohistochemistry confirmed the results of gene expression analysis for type I collagen, TNC, TNMD and COMP with no difference between the different isoforms of TGF-β. Although the presence of the TGF-β receptor in equine tendon cells was demonstrated in culture, exposure to TGF-β3 did not produce any statistically significant increases in expression of any of the tendon associated genes and neither isoform of TGF-β could induce changes in protein expression after 7 days of exposure. Interestingly, TNMD and THBS4 proteins were detected in the absence of mRNA (95). Maeda and co-workers investigated the conversion of mechanical force into TGF-β mediated biochemical signals utilizing a transgenic mouse strain that expressed the *SCX* promotor-driven GFP marker. TGF-β1,-2 and -3, and to a lesser extend GDF8, induced ScxGFP expression in primary tenocyte cultures isolated from adult ScxGFP mouse Achilles tendons. Inhibition of TGF-β type I receptor significantly decreased ScxGFP levels and *SCX* mRNA levels (214). All members of the TGF-β superfamily signal by binding to specific type I and type II serine/threonine kinase transmembrane receptors. Upon ligand binding, SMAD proteins are phosphorylated and translocated to the nucleus where they function as transcriptional regulators. SMAD 2 and 3 account for TGF-β and activin signaling, while SMAD 1, 5 and 8 mediate BMP signaling. Therefore, inhibition of SMAD3 signaling reduces the expression of ScxGFP and *SCX* mRNA levels as well (214).

4.9.5 Mechanical stimulation

As stated previously, embryonic tendon progenitor cell differentiation *in vivo* is regulated by a combination of mechanical and chemical factors (185). Little is known regarding the precise mechanics involved due to the challenges faced with trying to evaluate the effects of mechanical loading on developing tendon *in vivo*. It is assumed that static and dynamic forces are significant contributors to tendon formation during embryonic development (184), but the involvement of specific dynamic loading parameters (i.e. strain rate, strain magnitude, duration and frequency) is unknown (31).

Maeda et al. nicely demonstrated the importance of mechanical stimuli on tendon cell viability and expression of tendon associated genes *in vivo* and *in vitro* through the utilization of the adult ScxGFP mice. The acute loss of tensile loading in a complete transection model (of the Achilles tendon) led to a reduction in cell viability of around 70% and low ScxGFP expression in the remaining cells. Injection

of Botulinum toxin A in the triceps muscle of the ScxGFP mouse led to reversible gradual loss of tensile loading, resulting in a decreased muscle force (< 25% of normal) within 3 days, being associated with an 80% decrease in the number of tenocytes expressing ScxGFP as compared to the control group. Immunohistochemical analysis also showed a significant decrease in the abundance of type I collagen fibrils and *COMP* expression. An increase in cell death, determined through TUNEL-staining could not be detected in this model. At two weeks post injection of the Botulinum toxin A, the tendon began to recover. Numbers of ScxGFP-expressing tenocytes and the deposition of type I collagen and COMP increased. The regulating influence of mechanical forces on ScxGFP expression could also be demonstrated *in vitro*. Primary tenocyte cultures from adult ScxGFP mouse Achilles tendon showed a decrease in ScxGFP expression over time (<5% by day 6). ScxGFP expression was upregulated in tenocytes exposed to shear stress through the use of a microfluidic chamber system. Interestingly, as in TGF-β signaling, inhibition of the SMAD 2/3 pathway resulted in decreased expression of ScxGFP and *SCX* mRNA levels in mechanically stimulated tendon cells. As the group was also able to demonstrate that mechanical force resulted in an upregulation of TGF-β1 and *SCX* mRNA *in vitro*, they suggest that mechanical forces regulate *SCX* expression through activation of the TGF-β/SMAD 2/3 mediated pathway (214). In addition, Egerbacher et al. outlined the importance of homeostatic tension on tendon cell viability. Stress deprivation of rat tail tendon resulted in increased caspase-3 mRNA expression, increased positive staining for caspase-3 protein and increased apoptosis in tendon cells as compared to cyclically-loaded (3% strain at 0.17 Hz) and fresh control samples (69). Furthermore, Huismann et al. could demonstrate an upregulation in type I collagen and *SCX* gene expression in tenocytes culture in BioFlex™ subjected to mechanical stimulation as compared to unstimulated human tenocytes. The insertion of a rest period in cyclic stretching increased gene expression of type I collagen, *SCX* and *TGF-β* (342).

In line with the mechano-active nature of tendons and the possible influence of mechanical stimulation on phenotype and stage of differentiation in tendon-derived fibroblasts, a number of recent studies have investigated possible tenogenic effects of mechanical stimulation on stem cells. For example, Scott at al. demonstrated the influence of mechanical forces on *SCX* expression in a murine multi-potent cell line C3H10T1/2 grown in 3-D culture. Static load alone already led to a dramatic increase in *SCX* expression in a time dependent fashion. Expression levels of *SCX* were further increased through cyclic loading as compared to static loading. Additionally, *SCX* expression was similarly affected by increased strain levels and cycle number, as well as through the insertion of a rest period between each loading cycle (219).

Comparisons between 2-D and 3-D human BMSCs cultures grown in collagen gels revealed a higher expression level of *SCX* in the 3-D culture system. Varying the concentration of collagen in the scaffold appeared to have no detectable influence on gene expression. Comparing cyclic loading with 1% elongation at 1 Hz for 30 minutes per day to static loading for a total cultivation period of 7 days had no significant impact on the contraction levels of the collagen constructs. Cyclic loading was able to maintain the increased expression levels of *SCX*, which decreased over time in static loading. As such, the authors implicated dynamic stimulation as being a key factor in maintaining tenogenic differentiation in MSCs (218).

Raabe et al. investigated the tenogenic differentiation of equine ASCs embedded in a collagen matrix under the influence of tensile strain, growth differentiation factors and various oxygen tensions for a culture period of 3 weeks. The most optimal conditions were deemed to be an oxygen tension of 21%, tensile stimulation, and supplementation of GDF5 or -7. Interestingly, no viable cells were observed in their control group consisting of ASCs cultured for 3 weeks under mechanical stimulation, but without growth factors (343).

The culturing of tendon-derived fibroblasts and MSCs in 2-D or 3-D systems under mechanical stimulation offers the opportunity to explore the influence of mechanical cues, or the combination of mechanical and chemical cues, on cell phenotype and differentiation. Furthermore, it allows for characterization of the more authentic tenocyte *in vitro* and the evaluation of different treatment modalities for tendon injury *in vivo*. The 3-D culturing of tendon-derived fibroblasts generally results in higher expression levels of tendon related genes and therefore, most likely results in cells with a more advanced differentiation status than cells grown in 2-D culture. However, with regards to the effects of mechanical stimulation in these systems, it is difficult to draw any conclusions as to which settings and conditions are the most appropriate. This is hampered by the fact that there are numerous different commercial and custom made mechanical systems available for cell culture with a multitude of variables including strain rate, frequency, number of cycles, and rest periods. Furthermore, it has to be kept in mind that strains experienced by individual cells is not the same as that applied to tendon fascicles or to the scaffold used. Screen at al. were able to show that in tendon fascicles exposed to 8% strain, the local strain experienced by the cells did not appear to exceed 2% (344). In the BioFlex™ plate an equiaxial strain of 10% resulted in average strain experienced by the cells of approximately 3-5% (345). Characteristics of the scaffold material used may play a major role in the transfer of forces applied and those experienced by the cells. The importance of the properties of the scaffold material should not be underestimated, as interface

properties such as surface/ligand chemistry and density may govern differentiation and cell behavior (220).

Exposing human tenocytes to 3.5% cyclic strain at a frequency of 1 Hz for 2 hours caused increased expression of *MMP3* and *IL1β* (62). Cyclical stretching of human tenocytes from 4- to 12% at a frequency of 0.5 Hz caused an increased production of PGE_2, an inflammatory mediator of tendinopathy (64). Therefore, another topic that needs to be further addressed is the upregulation of MMPs and other inflammatory mediators triggered by mechanical stimulation and the significance thereof.

4.10 Aim of the study

Whilst reviewing the current literature, it became clear that our understanding of tendon development, tendon injury and possible strategies to enhance tendon healing is still very limited. The expansion of tenocytes *in vitro* is a helpful approach to analyzing tendon healing and to finding tools to support tendon regeneration. Maintaining a differentiated state of the cells under investigation is a major prerequisite for enabling meaningful conclusions to be reached. 2-D culture techniques are generally used to expand cells to sufficient numbers prior to use *in vivo*. However, there is growing evidence that tenocytes in monolayer culture undergo a process known as dedifferentiation and thereby loose some of their basic tenogenic characteristics such as the expression of tendon-associated genes and the typical spindle-shaped cell morphology. 3-D cell culture methods are thought to more closely mimic the *in vivo* cellular environment and thereby represent a potential means by which to stabilize the tenocyte phenotype and provide more intimate cell-cell and cell-matrix contacts compared with monolayer cultured tenocytes. Beside differences in cellular gene expression, 3-D culture systems may aid in the histologic identification of tenocytes. The hanging drop culture system has the capacity to generate spheroidal microtissues (MTs) composed of different cells types. In addition to their ability to enhance the differentiation of certain cell types, MTs also have the capacity to act as building blocks for tissue engineering applications. However, to the best of our knowledge, equine tenocytes have not yet been cultured as hanging drops and as such, their ability to adapt to a 3-D MT environment has not yet been characterized. Therefore, based on these criteria, the current Thesis sets out to address the following aims:

1. Characterize equine adult and fetal tenocytes in the MT format

a.) Maintain a differentiated state of equine tenocytes in the MT format

b.) Evaluate the MT format as a system for co-culture of equine tenocytes and MSCs

2. Evaluate the influence of mechanical stimulation on tenocytes in the MT format and the possibility to use MTs for tissue engineering

4.10.1 Hypothesis

The primary premise of the current study is that the maintenance of equine tenocytes in the hanging drop culture system will lead to the formation of MTs and subsequently enhance their tenogenic potential as determined by gene expression and histological analyses. Furthermore, the introduction of chemical and mechanical stimulation, along with MSC co-culture, is envisaged to have a beneficial influence on tenocyte differentiation in MTs.

5 Results

5.1 Use of biomimetic microtissue spheroids and specific growth factor supplementation to improve tenocyte differentiation and adaptation to a collagen-based scaffold *in vitro*

Biomaterials 69 (2015) 99–109

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Use of biomimetic microtissue spheroids and specific growth factor supplementation to improve tenocyte differentiation and adaptation to a collagen-based scaffold *in vitro*

Felix Theiss [a, b, c], Ali Mirsaidi [a, d], Rami Mhanna [e, f], Jan Kümmerle [a, b, c], Stephan Glanz [a, d], Gregor Bahrenberg [a, d], André N. Tiaden [a], Peter J. Richards [a, d, *]

[a] Center for Applied Biotechnology and Molecular Medicine (CABMM), University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
[b] Equine Department, Vetsuisse-Faculty, University of Zurich, Winterthurerstrasse 260, 8057 Zurich, Switzerland
[c] Graduate School for Cellular and Biomedical Sciences, University of Bern, 3012 Bern, Switzerland
[d] Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, 8057 Zurich, Switzerland
[e] Cartilage Engineering and Regeneration, ETH Zurich, 8093 Zurich, Switzerland
[f] American University of Beirut, Faculty of Engineering and Architecture, Riad El Solh, 1107 2020 Beirut, Lebanon

ARTICLE INFO

Article history:
Received 16 April 2015
Received in revised form
6 August 2015
Accepted 7 August 2015
Available online 10 August 2015

Keywords:
Tendon
Growth factors
TGF (transforming growth factor)
Microsphere
In vitro test

ABSTRACT

Tenocytes represent a valuable source of cells for the purposes of tendon tissue engineering and regenerative medicine and as such, should possess a high degree of tenogenic differentiation prior to their use *in vivo* in order to achieve maximal efficacy. In the current report, we identify an efficient means by which to maintain differentiated tenocytes *in vitro* by employing the hanging drop technique in combination with defined growth media supplements. Equine tenocytes retained a more differentiated state when cultured as scaffold-free microtissue spheroids in low serum-containing medium supplemented with L-ascorbic acid 2-phosphate, insulin and transforming growth factor (TGF)-β1. This was made evident by significant increases in the expression levels of pro-tenogenic markers collagen type I (*COL1A2*), collagen type III (*COL3A1*), scleraxis (*SCX*) and tenomodulin (*TNMD*), as well as by enhanced levels of collagen type I and tenomodulin protein. Furthermore, tenocytes cultured under these conditions demonstrated a typical spindle-like morphology and when embedded in collagen gels, became highly aligned with respect to the orientation of the collagen structure following their migration out from the microtissue spheroids. Our findings therefore provide evidence to support the use of a biomimetic microtissue approach to culturing tenocytes and that in combination with the defined growth media described, can improve their differentiation status and functional repopulation of collagen matrix.

1. Introduction

Tendon lesions in equine and human athletes are mainly the result of overstrain injury rather than percutaneous trauma, where the athletic discipline generally predisposes specific tendons to injuries. In the racehorse and event horses, injuries occur most frequently in the suspensory ligament and the superficial digital flexor tendon (SDFT) [1]. In humans, tendinopathy is most commonly diagnosed in the Achilles, patellar, rotator cuff and medial/lateral elbow tendons and accounts for 30–50% of all sports-related injuries [2]. As such, there is an ever growing need for more effective therapies with which to combat tendon injuries and the ensuing degeneration associated with such trauma. The use of cell-based tissue engineering approaches are fast emerging as alternative therapeutic strategies for the management of tendon injury both in humans and animals [3]. Indeed, a growing number of reports now exist in which multipotent stromal cells (MSCs) isolated from various sources have been successfully implemented in the treatment of both experimentally induced tendon defects [4–7], as well as in clinical cases of tendinopathy [8,9]. The use of MSCs primarily relies on their ability to differentiate into fully functional tendon cells, termed tenocytes. As such, investigators have also sought to utilize mature tenocytes directly as an

* Corresponding author. Bone and Stem Cell Research Group, Competence Center for Applied Biotechnology and Molecular Medicine, Room 13-L-86, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland.
E-mail address: peter.richards@cabmm.uzh.ch (P.J. Richards).

http://dx.doi.org/10.1016/j.biomaterials.2015.08.013
0142-9612/

additional means by which to regenerate tendon tissue as evidenced by the initiation of human clinical trials [source: ClinicalTrials.gov, Trial Number: NCT01343836 and source: ClinicalTrialsRegister.eu, Trial Number: 2010-021869-73] with the aim to evaluate the efficacy of autologous tenocytes in treating chronic tendinopathy.

Tenocytes are fibroblast-like cells derived from embryonic mesenchyme and form a three dimensional network of cell processes throughout the extracellular matrix linked by gap junctions [10]. These cell processes not only connect tenocytes with each other, but also enclose the collagen bundles. It is thought that this close relationship between tenocytes and collagen fibril bundles enables cellular load sensing and coordination of response to load. Characterization of tenocyte gene expression has revealed several markers considered to be essential for their development into fully functional tendon-forming cells, of which tenomodulin (TNMD) may be considered one of the most important and well studied [11–13]. TNMD is a type II transmembrane protein that is mainly expressed in dense connective tissues, and is generally regarded as being a late marker of tendon development [14]. *TNMD* gene expression is positively regulated during tendon development by scleraxis (SCX) [14,15], a transcription factor considered essential for efficient tendon differentiation [16]. However, both TNMD and SCX have also been detected in tissues from sources other than tendon, thereby bringing into question their reliability as specific markers of tendon [17]. Despite this fact, both TNMD and SCX still serve as a means by which to gauge the differentiation status of tenocytes isolated from tendon tissue.

It has previously been reported that *in vitro*, mature tenocytes have a reduced tendency to express *TNMD*, as well as collagen type 1 (*COL1*), and lose their elongated morphology when cultured for extended periods [18–20]. This so called dedifferentiation of cultured tenocytes is not only considered to be potentially detrimental to their efficiency as a cell-based therapy in tendon repair, but also to their usefulness as an *in vitro* cell system for developing and testing alternative treatments. In this regard, various studies have been undertaken with an aim to improving the genotype and phenotype of isolated tenocytes. Of critical importance in this regard, is the growth medium and its components used to maintain tenocytes in culture. Several investigators have identified transforming growth factor (TGF)-β and insulin-like growth factor (IGF)-1 as being potent inducers of tenogenic differentiation in both MSCs and tenocytes [21–26], allowing cells to maintain a tenogenic genotype and phenotype *in vitro*. Furthermore, the transfer of cultured tenocytes to high density, three-dimensional (3D) growth environments also appears to stimulate differentiation and prevent cellular dedifferentiation to some degree [21,25–27].

The primary focus of the present study was to determine whether equine tenocytes could generate self-assembled gravity-enforced 3D microtissue spheroids *in vitro*, and if so, could this improve their tenogenic differentiation status over that of tenocytes cultured under standard 2D conditions. Furthermore, various combinations of growth factor supplementation were evaluated in terms of their ability to influence the genotype and/or phenotype of equine tenocytes maintained as 2D or 3D cultures. Equine tenocytes cultured as hanging drops formed microtissue spheroids, and after 2 and 6 days of culture, displayed significantly greater levels of several recognized tendon cell markers as compared to monolayer cultures as determined by RT-qPCR. Moreover, the use of low serum growth media supplemented with TGF-β1 in combination with insulin and ascorbic acid enhanced these effects, and when used to stimulate microtissue-derived tenocytes embedded in collagen scaffolds, induced phenotypic features typical of differentiated tenocytes. Our findings therefore suggest that scaffold-free biomimetic microtissue spheroids, in combination with specific chemical compositions of growth media, may represent an efficient means by which to maintain equine tenocyte differentiation *in vitro*. Such a system may therefore allow for tendon cell and tissue biology to be studied *in vitro* using conditions that more closely simulate the *in vivo* situation and could potentially offer an alternative strategy for stimulating tendon tissue formation *in vivo*.

2. Materials and methods

2.1. Animals

Tenocytes were isolated from the superficial digital flexor tendon of Warm blood horses, aged between 2 and 4 years, which were naturally destroyed for clinical reasons other than orthopaedic disease and where owner consent was obtained.

2.2. Isolation and culture of equine tenocytes

Tenocytes were isolated in accordance with previously established methodologies [17,20]. The mid-metacarpal region of the superficial digital flexor tendon was harvested under sterile conditions. All tendons were free of pathology on clinical and post mortem examination. After removal of the peritendineum, tendon sections of approximately 1 cm in length were cut into 2 mm^3 pieces and subjected to 0.2% collagenase NB4 (Roche Diagnostics, Rotkreuz, Switzerland) and 0.3% dispase II (Roche Diagnostics) digestion overnight in phosphate-buffered saline (Life Technologies, Zug, Switzerland) containing 5% penicillin/streptomycin (Life Technologies) on an orbital shaker at 37 °C. Digested tissue was filtered through a 70 μm cell strainer, centrifuged and cells resuspended in normal growth medium (GM) consisting of Dulbecco's modified eagle medium (DMEM-high glucose) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (all from Life Technologies). Supernatant was replaced after 1 day and thereafter every 3–4 days with fresh GM and cells were used between passage 4 and 7 unless otherwise stated. The potential contamination of tenocyte cultures with MSCs was assessed through the use of osteogenic and adipogenic induction assays according to previously described methodologies [28]. Briefly, monolayer cultures of equine tenocytes were incubated for 14 days with either osteogenic or adipogenic induction medium and stained with Alizarin red or Oil Red O in order to assess osteogenic and adipogenic differentiation respectively. Additionally, MSCs isolated from equine adipose tissue were also incubated with either osteogenic or adipogenic induction medium and served as a positive multipotent cell control.

2.3. Stimulation of equine tenocytes

For 2D monolayer cultures, equine tenocytes seeded at 6×10^4 cells/cm^2 and stimulated with GM, or DMEM-high glucose supplemented with either; (i) 1% FBS and TGFβ1 (10 ng/ml; Peprotech, London, UK); (ii) 1% FBS, TGFβ-1 (10 ng/ml) and IGF-1 (50 ng/ml; Peprotech, London, UK) (termed tenogenic differentiation medium-I; TDM-I) or with; (iii) 1% FBS, 50 μM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 0.5 μg/ml insulin (all from Sigma–Aldrich, Buchs, Switzerland) and 10 ng/ml human TGF-β1 (termed tenogenic differentiation medium-II; TDM-II). In order to generate tenocyte (TC)-microtissue spheroids, cells were seeded as hanging drops in Terasaki plates (VWR International, Dietikon, Switzerland) in GM, TDM-I or TDM-II according to previously published methodologies [29]. Briefly, equine tenocytes were adjusted to 4×10^4 to 2×10^5 cells/ml in culture medium and a 25 μl cell suspension transferred to individual wells of a Terasaki plate. The plate was then sealed with a lid and inverted in order to

generate hanging drops and enable gravity-enforced TC-microtissue spheroid formation. Cells grown as either monolayers or as microtissue spheroids were then harvested at selected time points for further analysis.

2.4. Tenocyte outgrowth studies

The potential for tenocytes to grow out from microtissue spheroids structures was examined using both 2D and 3D culture systems. TC-microtissues were initially cultured for 6 days in hanging drops using either GM or TDM-II. In the case of 2D outgrowth studies, TC-microtissues were transferred to 48-well plates at 60 spheroids/well for up to 3 days, and cell outgrowth visualized at selected time points by phase contrast microscopy. For analysis of cell outgrowth under 3D culture conditions, TC-microtissues consisting of 5×10^3 cells were mixed with 0.8% bovine collagen (Koken Co., Tokyo, Japan) at 800 microtissues/ml and transferred to silicon molds containing support ridges in order to allow for effective anchoring of the collagen gel [30]. Collagen gels cultured with either GM or TDM-II were harvested at selected time points, fixed in 4% paraformaldehyde for 12 h and then embedded in paraffin wax and sections stained using hematoxylin and eosin.

2.5. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated and purified using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA (0.5 μg) was reverse transcribed to cDNA using superscript II (Life Technologies) and random hexanucleotide primers (Promega AG, Dübendorf, Switzerland). Quantification of mRNA expression was performed with TaqMan Gene Expression Assays (Life Technologies) specific for *COL1A2* (Ec03469522_m1), *COL2A1* (Ec03467390_m1), *COL3A1* (Ec03469743_m1), *SCX* (Ec03818452_s1), *TNMD* (Ec03467883_m1) and *SOX9* (Ec03469763_s1) using the StepOnePlus Real-Time PCR System (Life Technologies) and values normalized to *GAPDH* (Ec03210916_gH) and presented as $2^{-\Delta CT}$. Each 10 μl reaction consisted of 1× TaqMan Fast Universal PCR Master Mix (Life Technologies), 1× TaqMan Gene Expression Assay and 10 ng cDNA. All reactions were performed in fast optical 96-well reaction plates (Life Technologies) at 95 °C for 20 s and 40 cycles of 95 °C for 1 s and 60 °C for 20 s.

2.6. Histology

For hematoxylin and eosin staining, dewaxed paraffin sections (5 μm) were rehydrated and stained with Harris' hematoxylin (Sigma–Aldrich, Buchs, Switzerland) for 2 min, rinsed in tap water, and treated with Scott's tap water for a further 2 min. Following washing in tap water, sections were stained with eosin Y (Sigma–Aldrich) for 3 min, rinsed quickly in tap water, dehydrated and mounted in DPX (Sigma–Aldrich). For immunohistochemical analysis, rehydrated tissue sections were blocked with normal 10% rabbit serum for 30 min and then incubated with polyclonal goat anti-collagen type I (1:100; LabForce, Muttenz, Switzerland) for 1 h at 37 °C. Sections were then washed in PBS and incubated with biotinylated rabbit anti-goat IgG (1:200; Reactolab SA, Servion, Switzerland) for 1 h at 37 °C followed by washing and a further incubation for 30 min with Vectastain (Reactolab SA). Sections were then developed using 3,3′ diaminobenzidine tetrahydrochloride (DAB), counterstained with Harris' Hematoxylin and mounted in DPX.

For active caspase-3 staining of TC-microtissue spheroids, paraffin wax tissue sections were initially microwaved in Target Retrieval Solution (Dako, Zug, Switzerland) for a total of 20 min at 750 W and then counterstained with Hemalaun for 2 min. Endogenous peroxidase activity was quenched using 3% H_2O_2 and sections blocked for 10 min using Protein Block Serum-Free (Dako). Slides were then incubated with rabbit anti-human/mouse active caspase-3 (1:50; R&D Systems, Abingdon, UK) overnight at 4 °C. Staining was detected using the Envision System (Dako) and developed using 3-amino-9-ethylcarbazole (AEC) (Life Technologies). Sections were subsequently mounted in Kaiser's gelatin glycerin (Fluka, Buchs, Switzerland) and visualized by light microscopy. Fetal equine TC-microtissue spheroids were included as a positive control for use in the active caspase-3 staining assay based on the consistently high numbers of apoptotic fetal equine tenocytes observed in microtissues after 6 days of culture. Equine fetal tendon cells were obtained from the Laboratory of Cellular Therapy, Department of Musculoskeletal Medicine, University Hospital of Lausanne.

2.7. Immunoblotting

Protein was analyzed by SDS-PAGE using 4–15% precast Tris–HCl gels (Bio-Rad Laboratories AG, Cressier, Switzerland) under reducing conditions, and electroblotted onto PVDF membranes using the Trans-Blot Turbo blotting system (Bio-Rad Laboratories AG). After blocking in 5% fat-free milk, 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20 (TBS-T) for 1 h at room temperature, membranes were incubated overnight at 4 °C with rabbit anti-tenomodulin (1:1000; Abcam, Cambridge, UK) or mouse anti-tubulin (1:10′000; Sigma–Aldrich). After washing in TBS-T, membranes were incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit (1:10′000; LabForce) followed by incubation in SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Reinach, Switzerland) and exposure to X-ray film.

2.8. Statistical analysis

All statistical analyses were carried out using SPSS19.0 (SPSS Inc., Chicago, IL). Parametric analysis of normally distributed data was performed using one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple group comparisons. In all cases, a p-value of <0.05 was considered statistically significant, and all data were expressed as mean ± standard deviation (S.D.).

3. Results

3.1. Equine tenocytes form microtissue spheroids in hanging drop cultures

Equine tenocyte cultures were deemed to be free of contaminating MSCs based on the lack of any discernable levels of mineralization or oil droplet formation when subjected to culture conditions conducive to either osteogenesis or adipogenesis respectively (Supp. Fig. 1). The equine tenocytes utilized in the current study typically demonstrated a fibroblast-like morphology when cultured in 2D monolayers as determined by phase contrast microscopy (Fig. 1a and b). The randomness of their orientation became apparent as cells approached confluency (Fig. 1b). Following their incorporation into hanging drop cultures, equine tenocytes readily self-assembled into microtissue spheroids after 60 h of incubation (Fig. 1c–h). The capacity for equine tenocytes to form microtissue spheroids was largely dependent on the initial cell seeding density, where 2500 (Fig. 1e and f) to 5000 (Fig. 1g and h) cells was considered to be the optimal cell number for efficient

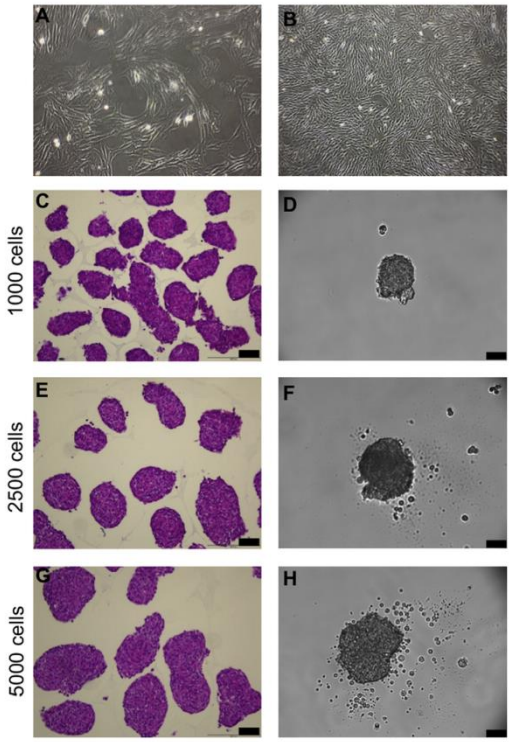


Fig. 1. Representative phase contrast images of sub-confluent (A) (magnification 20×) and confluent (B) (magnification 10×) equine tenocytes grown as 2D monolayer cultures. Representative images of hematoxylin and eosin stained paraffin wax sections of equine TC-microtissue spheroids after 60 h of culture using 1000 (C), 2500 (E) or 5000 (G) equine tenocytes. Phase contrast images of single hanging drops 60 h following seeding with 1000 (D), 2500 (F) or 5000 (H) equine tenocytes. Scale bar = 75 μm.

microtissue formation in the given time frame. During the first 4 days of hanging drop culture, cells were found randomly dispersed throughout the microtissue spheroids and lacked the characteristic fibroblast-like appearance of cells grown in 2D cultures (Fig. 2a and b) (Supp. Fig. 2a). However, by day 6, the majority of cells within the microtissue spheroids displayed an elongated phenotype and had become more ordered in appearance (Fig. 2c) (Supp. Fig. 2b). The capacity for equine tenocytes to tolerate the hanging drop 3D culture environment was made evident by the lack of caspase-3 activity in the microtissue spheroids at any of the time points tested (Fig. 2e–g). By contrast, high levels of caspase-3 activity were found to be a predominant feature of fetal equine TC-microtissues (Fig. 2d and h).

3.2. *Variations in culture media composition significantly influence TC-microtissue phenotype and genotype*

We next evaluated the effects of different growth media on the capacity of equine tenocytes to form microtissue spheroids over a 6 day period. In comparison to equine tenocytes cultured as hanging drops in the presence of GM alone (Fig. 3a *top panel*), there was a clear delay in the formation of single microtissue spheroids by cells grown in TDM-I (Fig. 3a *middle panel*) or TDM-II (Fig. 3a *lower panel*). In contrast to GM-treated cells, both TDM-I- and TDM-II-treated cells initially formed numerous smaller spheroids during the first 4 days of culture, but had generally coalesced by day 6 to form a single microtissue spheroid (Fig. 3b). Furthermore, in the case of cells cultured in TDM-II, there was also an appreciable accumulation of extracellular material surrounding the spheroid culture at days 4 and 6. In order to ascertain the influence of culture conditions on the tenocyte genotype, we analysed the expression levels of several tenogenic markers including *COL1A2*, *COL3A1*, *SCX*, and *TNMD*, as well as the chondrogenic markers *COL2A1* and *SOX9*. Comparisons were made between equine tenocytes that had been grown under 2D- or 3D-culture conditions with GM, TDM-I or TDM-II.

3.2.1. *2D cultures*

Cells cultured for 2 days in either TDM-I or TDM-II showed significant increases in *COL1A2*, *COL3A1*, *SCX* and *SOX9* expression levels as compared to cells in GM alone (Fig. 4a). However, expression levels of *TNMD* remained undetectable in all treatment groups. By day 6, cells cultured in TDM-II showed the greatest increases in gene expression for all makers tested, and represented the only culture condition in which *TNMD* expression could be detected. These results were therefore already suggestive of TDM-II as being a potential alternative to conventional growth medium for the maintenance of equine tenocytes in a more tenogenic-like state.

3.2.2. *3D cultures*

Treatment of TC-microtissue spheroids for 2 days with either TDM-I or TDM-II induced significant increases in *SCX* expression as compared to cultures receiving GM alone (Fig. 4b). TDM-II additionally induced significant increases in *COL3A1* and *SOX9* at this early time point. Expression levels of *COL1A2*, *COL3A1*, *SCX* and *TNMD* remained significantly elevated at day 6 in cultures treated with either TDM-I or TDM-II, with the greatest effects being observed in TDM-II-treated microtissues. In the case of *SOX9* expression, although TDM-I and TDM-II treatments resulted in significantly more gene expression in comparison to GM alone, *SOX9* mRNA levels were significantly reduced in all treatment groups at day 6 as compared to day 2 measurements ($p < 0.001$). Furthermore, incubation of TC-microtissues in low serum GM supplemented with TGFβ-1 alone for 6 days had no stimulatory effects on tenogenic marker expression, although *SOX9* expression was significantly upregulated (Supp. Fig. 3). Therefore, in addition to its beneficial effects on tenogenic gene expression in tenocyte monolayers, TDM-II also appeared to have a pro-tenogenic effect when used to culture tenocytes in 3D microtissue spheroids.

3.2.3. *2D vs. 3D cultures*

Comparisons in gene expression were also made between tenocytes grown in 2D or 3D culture systems. Microtissues cultured in GM alone for 2 days showed significant increases in *COL1A2*, *COL3A1*, *SCX* and *SOX9* expression levels when compared to tenocytes grown in monolayers (Fig. 5a). Similarly, microtissues stimulated with TDM-I or TDM-II demonstrated significant increases in *COL3A1*, *SCX* and *SOX9* expression as compared to their 2D counter parts. However, incorporation of tenocytes into 3D microtissues did not significantly enhance their capacity to express *COL1A2* in response to TDM-I or TDM-II. By contrast, *TNMD* mRNA was only detected in TC-microtissues, with the greatest levels being observed following TDM-II treatment. At day 6, overall gene expression was either unchanged or significantly reduced in GM-treated TC-microtissues as compared to tenocyte monolayers (Fig. 5b). Conversely, TC-microtissues cultured in TDM-I expressed greater levels of *COL1A2*, *COL3A1* and *TNMD* as compared to

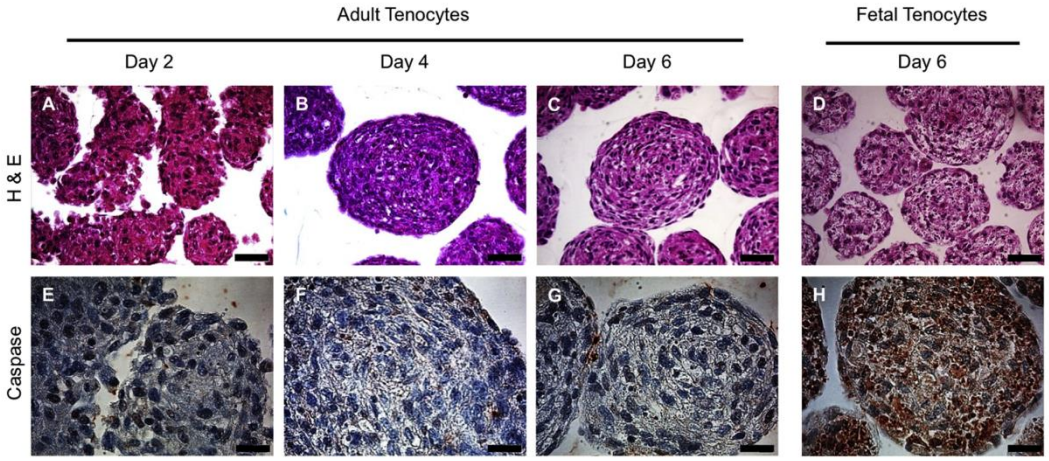


Fig. 2. Representative hematoxylin and eosin stained paraffin wax sections of equine TC-microtissue spheroids cultured for 2 days (A), 4 days (B) and 6 days (C) in GM and equine fetal tenocytes cultured for 6 days (D) in GM. Scale bar = 50 μm. Representative images of active caspase-3 staining (brown) in paraffin wax sections of equine TC-microtissue spheroids cultured for 2 (E), 4 (F) and 6 days (G) in GM. Equine fetal TC-microtissues cultured for 6 days (H) in GM were used as positive controls. Scale bar = 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tenocytes in monolayers. Similarly, TDM-II induced significant increases in *COL3A1*, *SCX* and *TNMD*, whilst significantly reducing the expression of the chondrogenic marker *SOX9*. The chondrogenic marker *COL2A1* was not detected in any of the culture conditions at either the 2 or 6 day time points. Taken together, these results demonstrate the effectiveness of culturing equine tenocytes as hanging drops in the presence of TDM-II for the purpose of maintaining cells in a more tenogenic-like state.

3.3. Tendon-associated protein production by TC-microtissues

Immunohistochemical analysis of TC-microtissue spheroids was undertaken to assess the impact of TDM-II on collagen type I protein expression. A noticeable increase in collagen type I was evident in equine TC-microtissue spheroids cultured in TDM-II for 6 days as compared to those cultured in GM alone (Fig. 6). In both cases, the majority of positive staining was observed at the periphery of the microtissue structures. These observations are in accordance with the differences observed in *COL1A2* gene expression between GM-treated and TDM-II-treated TC-microtissues, and thus supports the use of TDM-II as an efficient means by which to maintain equine tenocytes in 3D cultures. It should be noted that the agarose used to embed the microtissues for the purposes of histological processing also interacted non-specifically with the anti-COL1A antibody at the concentrations tested, thereby generating a diffuse background stain. In an attempt to further confirm the pro-tenogenic effects of the hanging drop culture and TDM-II stimulation, we analysed protein extracts for the presence of tenomodulin using Western blot. Although tenomodulin is predicted to have a molecular weight of 37 kDa, numerous sized bands ranging from 20 to 250 kDa were identified in TC-microtissue protein samples (Supp. Fig. 4). Similarly, numerous different sized protein bands were also detected in protein extracts from native equine tendon, with the most prominent bands being observed at 28, 34, 40, 65 and 250 kDa. Although it remains unclear as to the identity and specificity of these protein products, the 250 kDa protein band was only detected in samples taken from native equine tendon or equine TC-microtissues cultured in TDM-II.

3.4. Equine tenocyte outgrowth from microtissue spheroids

In order to assess the proliferative status of tenocytes cultured as hanging drops in GM or TDM-II, 6-day-old TC-microtissues were transferred to tissue culture plates and visualized by phase contrast microscopy over the course of 3 days (Fig. 7). During the first 24 h of culture, cells were observed to migrate out from the microtissues onto the plastic surface of the tissue culture plate (Fig. 7a and b). Furthermore, cells previously cultured as TC-microtissues in the presence of TDM-II (Fig. 7b) appeared to have a more spindle shaped, tenocyte-like appearance as compared to those cultured in GM alone (Fig. 7a). By day 3, the number of cells populating the tissue culture surface had noticeably increased in both GM- and TDM-II-treated TC-microtissue cultures (Fig. 7c and d). Cells emanating from the TDM-II-treated TC-microtissues continued to demonstrate morphology reminiscent of tenocytes (Fig. 7d) in comparison to the rounded cell appearance of the GM-treated TC-microtissues (Fig. 7c). The ability of tenocytes to migrate out from the microtissue spheroids was further examined using collagen gels as a means by which to simulate a more physiologically relevant 3D environment. Macroscopic observations of TC-microtissue seeded collagen gels revealed noticeable amounts of matrix contraction in gels containing the TDM-II-treated microtissues (Supp. Fig. 5). Gel contraction was already evident after 2 days of TC-microtissue seeding and continued to increase over the next 3 days. By contrast, no such contraction was seen in gels seeded with GM-treated microtissues at any of the time points tested. Similarly to the 2D culture system, migration of tenocytes out from the microtissues was observed for both GM- and TDM-II-treated cells within the first 24 h (Supp. Fig. 6) and continued to increase over the next 4 days in association with a gradual reduction in microtissue structure (Fig. 8). However, in contrast to collagen gels containing GM-treated microtissues, gels seeded with TDM-II-treated microtissues contained numerous, well-orientated cells aligned in the direction of the contracted collagen matrix (Fig. 8b, d and f). Moreover, by day 5 after the initial TC-microtissue seeding, collagen gels containing TDM-II-treated microtissues started to take on a

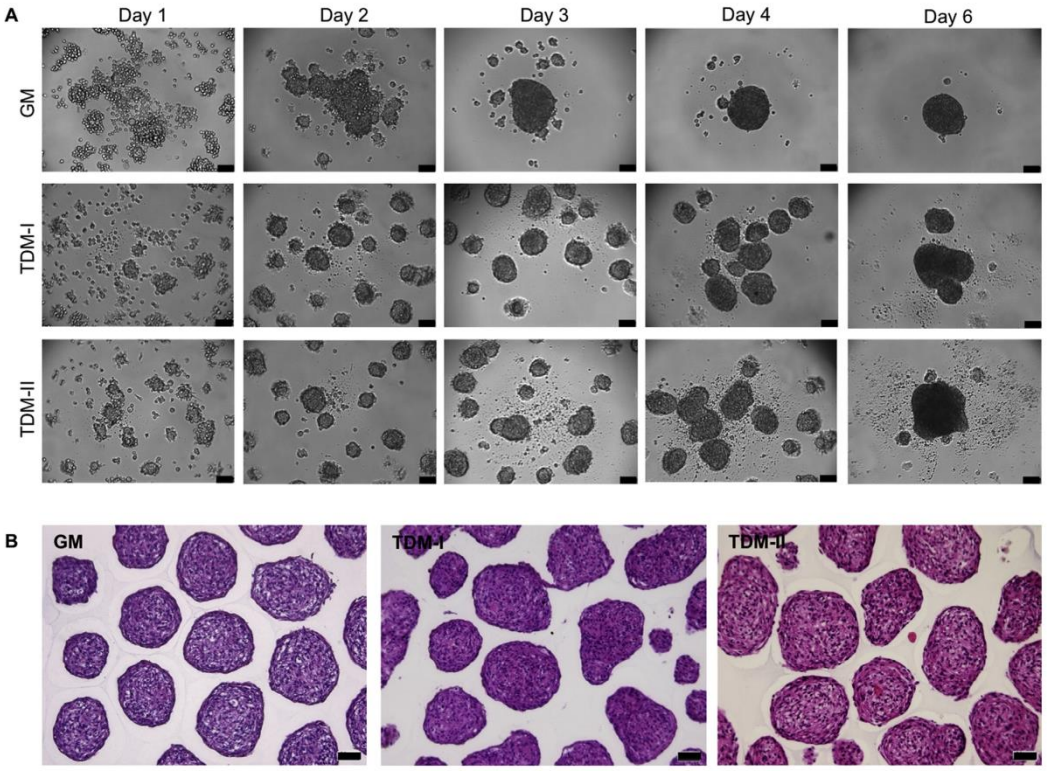


Fig. 3. (A) Representative phase contrast images of microtissue spheroid formation in single hanging drops of tenocyte cultures treated for up to 6 days with either growth medium (GM) alone, tenogenic differentiation medium I (TDM-I) or tenogenic differentiation medium II (TDM-II). Scale bar = 75 μm. (B) Representative images of hematoxylin stained paraffin wax sections of TC-microtissue spheroids at day 6 following treatment with GM, TDM-I or TDM-II. Scale bar = 50 μm.

more native tendon-like appearance, with cells displaying the characteristic spindle-like morphology dispersed throughout the matrix (Fig. 8f) (Supp. Fig. 7).

4. Discussion

Tendons are highly specialized structures composed mainly of collagen type I, whose principal function is to transmit muscular forces to bone and thereby enable stabilization and movement of joints. Furthermore, in horses, specific tendons can function as elastic energy storage structures, allowing for an athletic and energy efficient gait [31]. Repetitive overloading of tendons can result in cumulative micro-damage with degenerative changes of the ECM [32,33], and these alterations are invariably associated with physical disruption of fibres, cross-links and matrix proteins [34], being secondary to impaired tenocyte metabolism possibly also due to hyperthermic insults [35] or hypoxic cell injury [36]. In all cases, the resident cell population of the tendon fails to repair this cumulative micro-damage. As such, efforts are now being made to utilize various cell-based tissue engineering approaches to treat tendon injuries, with an aim to restoring tissue function as well as preventing associated degenerative changes.

As the primary source of collagen production in tendons, tenocytes have long been considered as a potential tissue engineering strategy to facilitate tendon repair and regeneration. Indeed, several studies now exist in which tenocytes have been successfully used for the purpose of augmenting tendon repair in both animal and human subjects [37–40]. However, in order to generate sufficient numbers of tenocytes for use *in vivo*, cells must first be expanded in culture. Although the isolation and expansion of tenocytes is nowadays a relatively standardized procedure, the choice of growth conditions under which tenocytes are cultivated can have a significant impact on their differentiation status and hence their functional capacity to repair tendon tissue. This has been highlighted in several studies in which the expression of tendon-associated markers, including *COL1*, *COL3*, *SCX* and *TNMD*, were shown to be downregulated in tenocytes cultured for extended periods [19,20]. These limitations however, could be overcome to some degree by incorporation of tenocytes into 3D culture systems [25,27,41] or through the addition of specific growth factors [22,24,25]. In the current report, we initially investigated the effectiveness of culturing equine tenocytes as hanging drops as a means by which to generate scaffold-free 3D microtissue spheroids. Tenocytes readily aggregated together and formed well-organized, viable microtissue spheroid structures within 6 days of culture. Furthermore, cells within these late stage

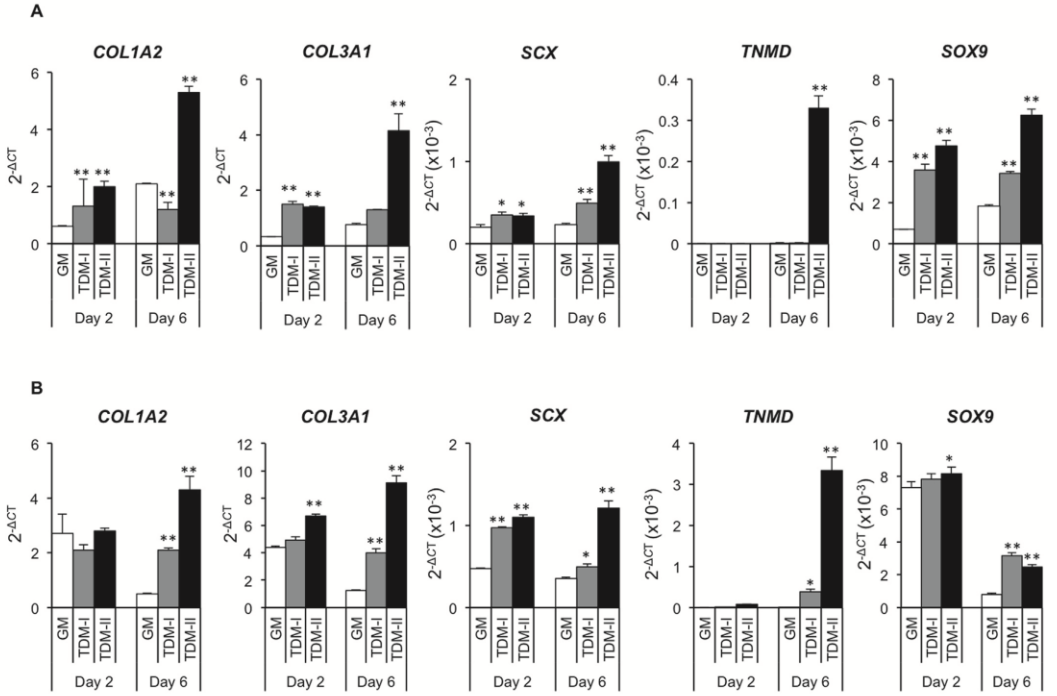


Fig. 4. qRT-PCR analysis of *COL1A2*, *COL3A1*, *SCX*, *TNMD* and *SOX9* in equine tenocytes cultured in 2D monolayers (A) or 3D microtissue spheroids (B) for 2 and 6 days. In each case, gene expression in equine tenocytes cultured in growth medium (GM) alone was compared to cells cultured in either tenogenic differentiation medium I (TDM-I) or tenogenic differentiation medium II (TDM-II) at each of the time points tested. Data was normalized to *GAPDH* and is presented as $2^{-\Delta CT}$. *$p < 0.05$, **$p < 0.01$ as compared to tenocytes cultured in GM. Significance was determined by one-way ANOVA and Tukey's post-hoc test. (±S.D. triplicates).

cultures appeared to have an elongated fibroblast-like morphology and demonstrated a basic cell alignment growth pattern. However, despite tenocyte-derived microtissue spheroids demonstrating early significant increases in tendon-associated markers such as *COL1A2*, *COL3A1* and *SCX* when compared to tenocyte monolayers, they were unable to maintain these elevated levels after prolonged culture times. Interestingly, the pro-chondrogenic marker *SOX9* was significantly reduced in these late-stage 3D cultures as compared to cells grown in monolayers. Moreover, in neither case were tenocytes observed to express *TNMD* or the chondrogenic-associated marker *COL2A1* at any of the time points tested. Taken together, these initial observations suggested that scaffold-free microtissue spheroid cultures may indeed have some immediate beneficial impact on tenocyte differentiation, although lacked the ability to maintain this effect over extended periods in culture.

In order to better optimize the tenogenic status of tenocytes cultured under either 2D or 3D conditions, we next focused our attention on the growth media used in each system. Findings from recent studies have strongly hinted at the use of TGF-β and IGF-1, or combinations thereof, to enhance and possibly even maintain tenocyte differentiation during extended periods of cultivation. Caliari et al. [22] demonstrated that IGF-1 at concentrations ranging from 10 to 200 ng/ml was capable of significantly enhancing *COL1A2*, *COL3A1* and decorin (*DCN*) gene expression in equine tenocytes cultured in collagen scaffolds in the absence of serum for 7 days. By contrast, a study performed by Qiu et al. [24] demonstrated that IGF-1 (50 ng/ml) alone was unable to significantly influence *COL1*, *SCX* or *TNMD* expression in human tenocytes. However, incubation of cells with TGF-β3 (10 ng/ml), or TGF-β3 and IGF-1 together, significantly enhanced gene expression above that of control cultures incubated in the presence of serum-supplemented growth medium. In support of this, studies investigating the tenogenic potential of primary canine MSCs revealed that combinations of TGF-β1 and IGF-1 (5 ng/ml of each) were sufficient to induce the expression of *COL1*, *COL3*, *DCN*, *SCX* and *TNMD* after 7 and 14 days in combination with high density 3D culture conditions [26]. Similarly, a more recent report by Barsby et al. [21] has confirmed a synergistic effect between 3D growth environments and the addition of TGF-β3 (20 ng/ml) in the tenogenic differentiation of equine embryonic stem cells (ESCs). Due to its high homology to IGF-1, insulin has also been evaluated for its ability to influence tenogenic differentiation and has proven to be a potent inducer of tenocyte formation in human MSC cultures [42]. Additionally, ascorbic acid has been identified as having a beneficial impact on protein synthesis and cell viability of tendon organ cultures [43]. Therefore, based on these previous findings, we elected to assess the effects of low-serum media containing human TGF-β1 (10 ng/ml) in combination with either human IGF-1 (50 ng/ml) (termed TDM-I), or human insulin and L-ascorbic acid 2-phosphate (termed TDM-II) on tenocyte differentiation in either 2D or 3D culture systems. Treatment of tenocytes in 2D with TDM-I had a moderate effect on most of the genetic markers tested, with the

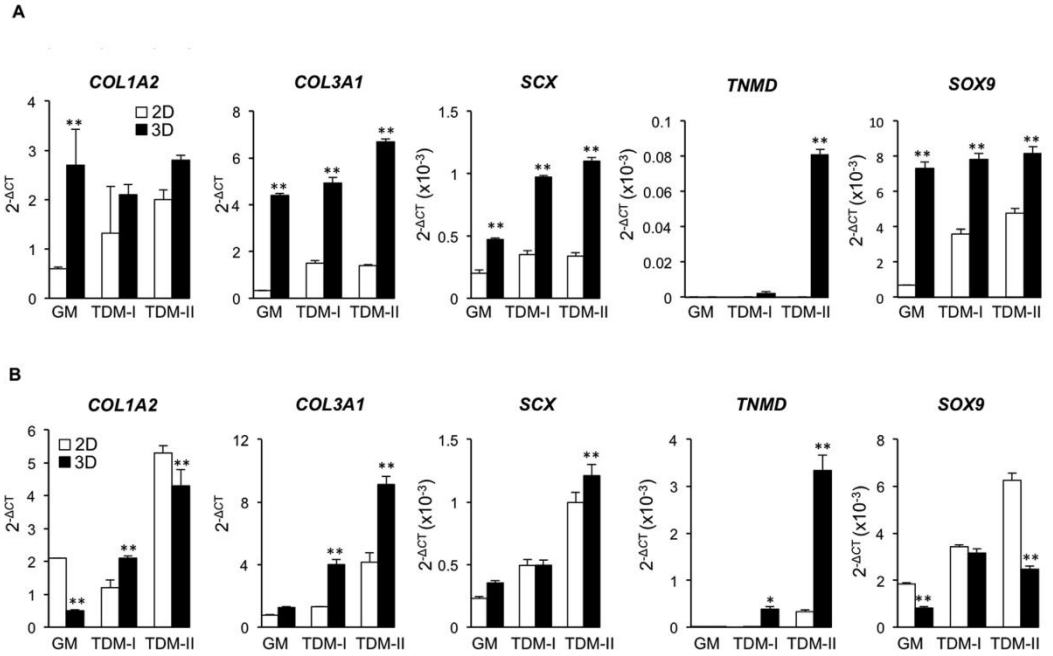


Fig. 5. qRT-PCR analysis of *COL1A2*, *COL3A1*, *SCX*, *TNMD* and *SOX9* in 2D or 3D equine tenocyte cultures at 2 (A) and 6 days (B) after seeding. Comparisons were made between tenocyte monolayers (2D) and microtissue spheroids (3D) when cultured in either growth medium (GM), tenogenic differentiation medium I (TDM-I) or tenogenic differentiation medium II (TDM-II). In each case, gene expression in equine tenocytes cultured in 2D was compared to those cultured in 3D, and data normalized to *GAPDH* and presented as $2^{-\Delta CT}$. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to tenocytes cultured in 2D monolayers. Significance was determined by one-way ANOVA and Tukey's post-hoc test. (±S.D. triplicates).

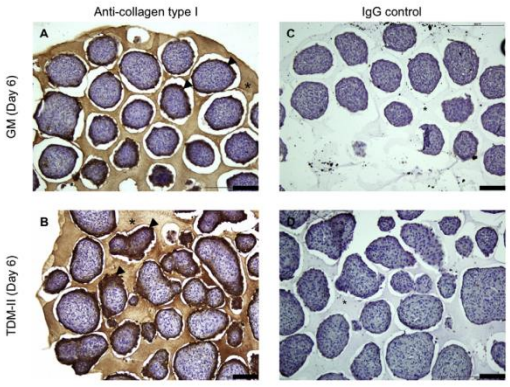


Fig. 6. Representative micrographs of anti-collagen type I stained paraffin wax sections of equine tenocytes cultured in hanging drops for 6 days in either GM (A) or TDM-II (B). Collagen type I was visualized using horseradish peroxidase-diaminodenzidine (dark brown) and sections counterstained with hematoxylin (blue). Examples of positive staining for collagen type I are indicated by arrowheads. (C–D) Representative micrographs of control paraffin wax sections in which the anti-COL1A primary antibody was replaced with normal IgG. *Asterisks*, agarose in which microtissues were embedded. Scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

majority of genes being significantly increased within the first 2 days of culture as compared to cells cultured in growth medium alone. However, induction of *TNMD* expression was not observed at any time point, and *COL1A2* and *COL3A1* expression levels were reduced by day 6. With regards to 3D cultures, early significant increases in gene expression were limited to *SCX* only, but at later

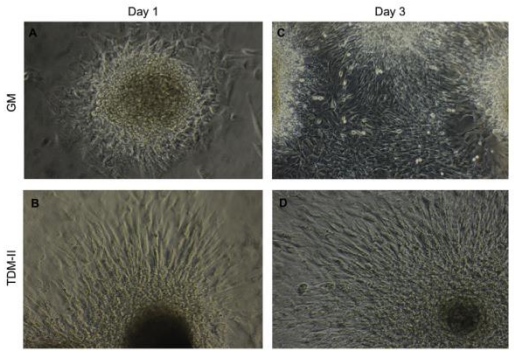


Fig. 7. Phase contrast micrographs demonstrating tenocyte outgrowth from microtissue spheroids under 2D culture conditions. Equine tenocytes were cultured as hanging drops for 6 days in GM or TDM-II and transferred to 48-well plates at 60 spheroids/well for 1 (A, B) and 3 (C, D) days. Magnification = 20×.

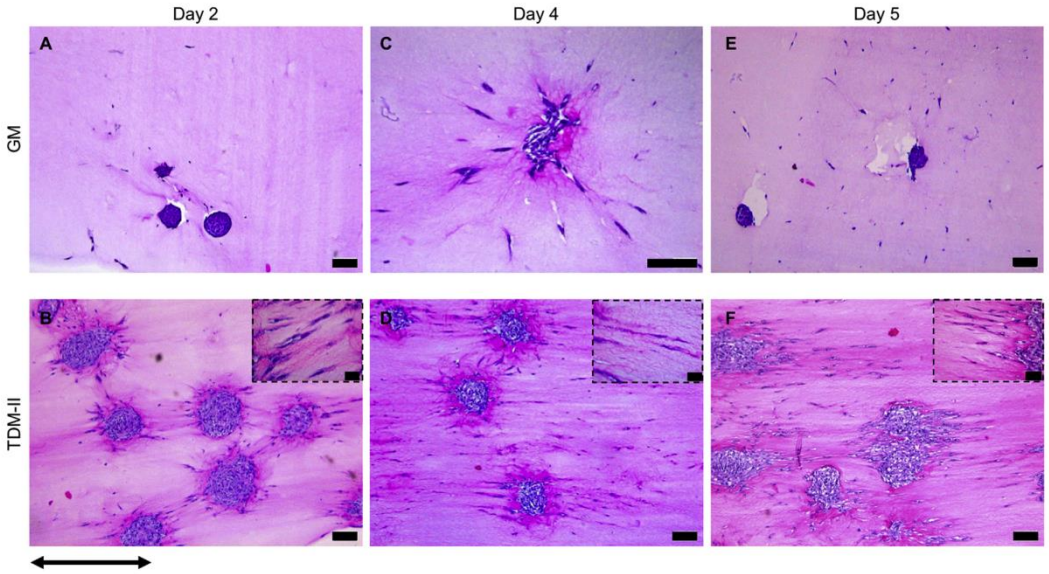


Fig. 8. Outgrowth of equine tenocytes from TC-microtissue and their alignment in static 3D collagen constructs. TC-microtissues were prepared in hanging drops for 6 days in either GM or TDM-II and then embedded in 0.8% collagen matrix. Representative micrographs of hematoxylin and eosin stained paraffin wax sections of gel constructs at 2 (A, B), 4 (C, D) and 5 (E, F) days after TC-microtissue seeding of GM (A, C, E) or TDM-II (B, D, F) treated cultures. Scale bar = 100 μm. *Inset*, scale bar = 20 μm. *Arrow*, orientation of collagen gels based on their positioning in anchored silicon molds.

stages included increases in *COL1A2*, *COL3A1*, *SCX*, *TNMD* and *SOX9*. In comparison to 2D cultures, late stage 3D cultures demonstrated modest benefits in terms of *COL1A2*, *COL3A1* and *TNMD* expression, with similar levels of *SOX9* being observed. These observations therefore go someway to supporting the concept of culturing tenocytes under 3D conditions in the presence of TGF-β and IGF-1 in order to enhance and maintain a tendon like genotype.

With regards to tenogenic gene expression, the most pronounced effects were observed in TDM-II, where IGF-1 had been replaced by insulin and L-ascorbic acid 2-phosphate. Regardless of which system was used, cultivation in TDM-II resulted in marked increases in the expression of all tenogenic gene markers in late stage cultures. Furthermore, in comparison to 2D cultures, TC-microtissue spheroids examined at day 6 demonstrated significantly enhanced expression levels of *COL3A1*, *SCX* and *TNMD*, with a noticeable decrease in the expression of the chondrogenic marker *SOX9*, and a complete absence of *COL2A1*. Moreover, not only were the expression levels of tenogenic markers maintained in this medium, but were also in some cases enhanced at the later stages. Clearly therefore, the combination of TGF-β1, insulin and ascorbic acid appears to represent a novel supplement for the purpose of enhancing and maintaining a tendon-like genotype in 3D tenocyte cultures, without inducing chondrocyte-associated gene expression. It should be noted however, that *SOX9* expression was increased by both TDM-I and TDM-II treatments in 2D and 3D culture systems. The transcription factor SOX9 is recognized as playing a pivotal role in regulating chondrocyte differentiation and as such, is considered to be a reliable marker of chondrogenesis [44]. Therefore, its upregulation in tenocyte cultures in response to TDM-I or TDM-II could imply alterations in tenocyte differentiation towards a more chondrogenic-like state. Certainly, over expression of *SOX9* in cultured tenocytes has been shown to result in decreases in the expression levels of *SCX* and *TNMD* and increases in *COL2A1* [45]. Moreover, forced expression of *SOX9* in chick tendon tissue can lead to ectopic cartilage formation [45]. However, despite our observations that *SOX9* expression levels were significantly elevated in 2D and 3D tenocyte cultures treated with either TDM-I or TDM-II, the expression levels of *SCX* and *TNMD*, where expressed, did not decrease. More importantly, *COL2A1* expression remained below detection levels at all time points, regardless of treatment regime. It therefore seems unlikely that the addition of TDM-I or TDM-II had any profound influence on the chondrogenic status of the equine tenocytes used in the current study. By comparison, tenogenic markers were noticeably absent in 3D cultures treated with TGFβ-1 alone and expression levels of *SOX9* remained significantly elevated, thereby indicating media supplemented solely with TGFβ-1 to be unsuitable for supporting the tenogenic differentiation status of equine tenocytes in 3D microtissue spheroids.

In accordance with these findings, we could demonstrate that collagen type I was also increased at the protein level in TC-microtissue spheroids cultured in the presence of TDM-II. Furthermore, when assessing protein lysates for the presence of TNMD, we detected an additional, high molecular weight protein not observed in TC-microtissue spheroids treated with growth medium alone. It is unclear as to why TNMD was detected in tenocyte cultures treated with growth medium at all, as qRT-PCR analysis failed to detect any *TNMD* mRNA transcript. However, a similar finding was also reported by Barsby et al. [46] in which TNMD protein was observed in TGF-β-treated equine ESC cultures in the absence of any detectable levels of *TNMD* mRNA. The cause for such inconsistencies was accredited to disparities between TNMD protein turnover and mRNA degradation rates. Therefore, it seems plausible that the 250 kDa band observed in the current study represents a high molecular weight variant of TNMD, being

present in native tendon tissue, as well as being induced in TC-microtissue spheroids following stimulation with TDM-II. However, the relevance of this particular protein species with regards to the normal functioning of tendons remains to be determined. In a final series of experiments, we assessed the potential of tenocytes within microtissue spheroids to migrate out from the tissue structures and to re-populate 2D or 3D culture environments. This was based on the assumption that should such tissue constructs be considered as a viable *in vivo* treatment strategy, then the cells contained within the tissue spheroids would be required to grow out and invade the surrounding host tissue in order to mediate their therapeutic effects. Indeed, we could demonstrate that TC-microtissue spheroids treated with TDM-II were capable of growing out from the tissues and could proliferate on a 2D culture surface whilst maintaining a tenocyte-like morphology. Moreover, when transferred to collagen gels, microtissue-derived cells treated with TDM-II also had the capacity to promote contraction of the collagen matrix and were observed to independently align with respect to the orientation of the collagen structure following microtissue outgrowth.

5. Conclusions

Taken together, our findings demonstrate the beneficial effects of culturing tenocytes as gravity-enforced microtissue spheroids and supports the use of low-serum containing growth medium in combination with TGF-β1, insulin and ascorbic acid as an effective means by which to maintain the tenogenic differentiation status of cultured tenocytes. Such a system not only offers the potential for tendon cell and tissue biology to be studied *in vitro* using conditions that more closely simulate the *in vivo* situation, but also raises the interesting possibility of whether this could be directly incorporated into future *in vivo* tissue engineering strategies for the purpose of more effectively treating tendon disorders.

Acknowledgements

This study was funded in part by the Stiftung Forschung für das Pferd. SG and GB were supported by SNSF grants 31003A_134935 and 31003A_156313. AM and NAT were supported by the Uniscientia Foundation. The authors would like to thank Prof. Dr. Brigitte von Rechenberg (Musculoskeletal Research Unit, Equine Department, Vetsuisse-Faculty, University of Zurich) and Dr. Silke Kalchofner-Mark (CABMM, University of Zurich) for their scientific input and support, Prof. Lee Ann Laurent-Applegate (Laboratory of Cellular Therapy, Department of Musculoskeletal Medicine, University Hospital Lausanne) for supplying equine fetal tenocytes and Katalin Zlinszky (Musculoskeletal Research Unit, Equine Department, Vetsuisse-Faculty, University of Zurich) for performing the caspase activity assay.

Appendix A. Supplementary data

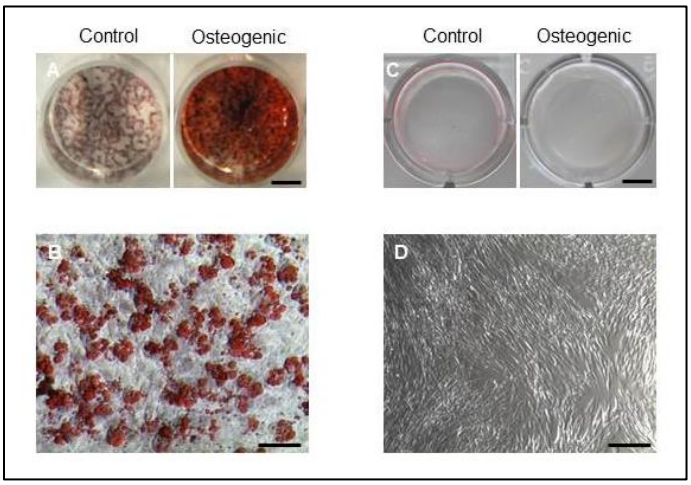
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.08.013.

References

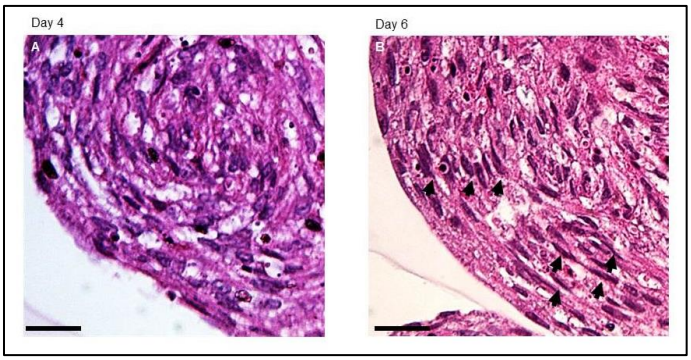
[1] R.B. Williams, L.S. Harkins, C.J. Hammond, J.L. Wood, Racehorse injuries, clinical problems and fatalities recorded on British racecourses from flat racing and National Hunt racing during 1996, 1997 and 1998, Equine Vet. J. 33 (2001) 478–486.
[2] P.P. Lui, N. Maffulli, C. Rolf, R.K. Smith, What are the validated animal models for tendinopathy? Scand. J. Med. Sci. Sports 21 (2011) 3–17.
[3] D. Gaspar, K. Spanoudes, C. Holladay, A. Pandit, D. Zeugolis, Progress in cell-based therapies for tendon repair, Adv. Drug Deliv. Rev. 84 (2015) 240–256.
[4] D. Deng, W. Wang, B. Wang, P. Zhang, G. Zhou, W.J. Zhang, Y. Cao, W. Liu, Repair of Achilles tendon defect with autologous ASCs engineered tendon in a rabbit model, Biomaterials 35 (2014) 8801–8809.
[5] J. Zhang, B. Li, J.H. Wang, The role of engineered tendon matrix in the stemness of tendon stem cells in vitro and the promotion of tendon-like tissue formation in vivo, Biomaterials 32 (2011) 6972–6981.
[6] J.Y. Lee, Z. Zhou, P.J. Taub, M. Ramcharan, Y. Li, T. Akinbiyi, E.R. Maharam, et al., BMP-12 treatment of adult mesenchymal stem cells in vitro augments tendon-like tissue formation and defect repair in vivo, PLoS One 6 (2011) e17531.
[7] J.L. Chen, Z. Yin, W.L. Shen, X. Chen, B.C. Heng, X.H. Zou, H.W. Ouyang, Efficacy of hESC-MSCs in knitted silk-collagen scaffold for tendon tissue engineering and their roles, Biomaterials 31 (2010) 9438–9451.
[8] R.K. Smith, N.J. Werling, S.G. Dakin, R. Alam, A.E. Goodship, J. Dudhia, Beneficial effects of autologous bone marrow-derived mesenchymal stem cells in naturally occurring tendinopathy, PLoS One 8 (2013) e75697.
[9] J.J. Albano, R.W. Alexander, Autologous fat grafting as a mesenchymal stem cell source and living bioscaffold in a patellar tendon tear, Clin. J. Sport Med. 21 (2011) 359–361.
[10] C.M. McNeilly, A.J. Banes, M. Benjamin, J.R. Ralphs, Tendon cells in vivo form a three dimensional network of cell processes linked by gap junctions, J. Anat. 189 (1996) 593–600.
[11] J. Qi, J.M. Dmochowski, A.N. Banes, M. Tsuzaki, D. Bynum, M. Patterson, A. Creighton, et al., Differential expression and cellular localization of novel isoforms of the tendon biomarker tenomodulin, J. Appl. Physiol. 113 (2012) 861–871.
[12] D. Docheva, E.B. Hunziker, R. Fässler, O. Brandau, Tenomodulin is necessary for tenocyte proliferation and tendon maturation, Mol. Cell Biol. 25 (2005) 699–705.
[13] P. Alberton, S. Dex, C. Popov, C. Shukunami, M. Schieker, D. Docheva, Loss of tenomodulin results in reduced self-renewal and augmented senescence of tendon stem/progenitor cells, Stem Cells Dev. 42 (2015) 597–609.
[14] C. Shukunami, A. Takimoto, M. Oro, Y. Hiraki, Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes, Dev. Biol. 298 (2006) 234–247.
[15] R. Schweitzer, E. Zelzer, T. Volk, Connecting muscles to tendons: tendons and musculoskeletal development in flies and vertebrates, Development 137 (2010) 2807–2817.
[16] N.D. Murchison, B.A. Price, D.A. Conner, D.R. Keene, E.N. Olson, C.J. Tabin, R. Schweitzer, Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons, Development 134 (2007) 2697–2708.
[17] S.E. Taylor, A. Vaughan-Thomas, D.N. Clements, G. Pinchbeck, L.C. Macrory, R.K. Smith, P.D. Clegg, Gene expression markers of tendon fibroblasts in normal and diseased tissue compared to monolayer and three dimensional culture systems, BMC Musculoskelet. Disord. 10 (2009) 27.
[18] L. Yao, C.S. Bestwick, L.A. Bestwick, Phenotypic drift in human tenocyte culture, Tissue Eng. 12 (2006) 1843–1849.
[19] A.D. Mazzocca, D. Chowaniec, M.B. McCarthy, K. Beitzel, M.P. Cote, W. McKinnon, R. Arciero, In vitro changes in human tenocyte cultures obtained from proximal biceps tendon: multiple passages result in changes in routine cell markers, Knee Surg. Sports Traumatol. Arthrosc. 20 (2012) 1666–1672.
[20] J. Zhu, J. Li, B. Wang, W.J. Zhang, G. Zhou, Y. Cao, W. Liu, The regulation of phenotype of cultured tenocytes by microgrooved surface structure, Biomaterials 31 (2010) 6952–6958.
[21] T. Barsby, E.P. Bavin, D.J. Guest, Three-dimensional culture and transforming growth factor beta3 synergistically promote tenogenic differentiation of equine embryo-derived stem cells, Tissue Eng. Part A 20 (2014) 2604–2613.
[22] S.R. Caliari, B.A. Harley, Composite growth factor supplementation strategies to enhance tenocyte bioactivity in aligned collagen-GAG scaffolds, Tissue Eng. Part A 19 (2013) 1100–1112.
[23] M.A. Costa, C. Wu, B.V. Pham, A.K. Chong, H.M. Pham, J. Chang, Tissue engineering of flexor tendons: optimization of tenocyte proliferation using growth factor supplementation, Tissue Eng. 12 (2006) 1937–1943.
[24] Y. Qiu, X. Wang, Y. Zhang, A.J. Carr, L. Zhu, Z. Xia, A. Sabokbar, Development of a refined tenocyte differentiation culture technique for tendon tissue engineering, Cells Tissues Organs 197 (2013) 27–36.
[25] Y. Qiu, X. Wang, Y. Zhang, A.J. Carr, L. Zhu, Z. Xia, A. Sabokbar, In vitro two-dimensional and three-dimensional tenocyte culture for tendon tissue engineering, J. Tissue Eng. Regen. Med. (2013), http://dx.doi.org/10.1002/term.1791.
[26] P.R. Schneider, C. Buhrmann, A. Mobasheri, U. Matis, M. Shakibaei, Three-dimensional high-density co-culture with primary tenocytes induces tenogenic differentiation in mesenchymal stem cells, J. Orthop. Res. 29 (2011) 1351–1360.
[27] G. Schulze-Tanzil, A. Mobasheri, P.D. Clegg, J. Sendzik, T. John, M. Shakibaei, Cultivation of human tenocytes in high-density culture, Histochem Cell Biol. 122 (2004) 219–228.
[28] J. Braun, A. Hack, M. Weis-Klemm, S. Conrad, S. Treml, K. Kohler, U. Walliser, et al., Evaluation of the osteogenic and chondrogenic differentiation capacities of equine adipose tissue-derived mesenchymal stem cells, Am. J. Vet. Res. 71 (2010) 1228–1236.
[29] A. Mirsaidi, A.N. Tiaden, P.J. Richards, Preparation and osteogenic differentiation of scaffold-free mouse adipose-derived stromal cell microtissue spheroids (ASC-MT), Curr. Protoc. Stem Cell Biol. 27 (2013). Unit 2B.5.

[30] R. Mhanna, E. Öztürk, P. Schlink, M. Zenobi-Wong, Probing the microenvironmental conditions for induction of superficial zone protein expression, Osteoarthr. Cartil. 21 (2013) 1924–1932.
[31] R.K. Smith, Mesenchymal stem cell therapy for equine tendinopathy, Disabil. Rehabil. 30 (2008) 1752–1758.
[32] R. Smith, W. McIlwraith, R. Schweitzer, K. Kadler, J. Cook, B. Caterson, S. Dakin, et al., Advances in the understanding of tendinopathies: a report on the Second Havemeyer Workshop on equine tendon disease, Equine Vet. J. 46 (2014) 4–9.
[33] J.H. Shepherd, G.P. Riley, H.R. Screen, Early stage fatigue damage occurs in bovine tendon fascicles in the absence of changes in mechanics at either the gross or micro-structural level, J. Mech. Behav. Biomed. Mater. 38 (2014) 163–172.
[34] C.S. Avella, R.K.W. Smith, Diagnosis and management of tendon and ligament disorders, in: J.A. Auer, J.A. Stick (Eds.), Equine Surgery, fourth ed., Elsevier Saunders, St. Louis, MO, USA, 2012, pp. 1157–1179.
[35] H.L. Birch, A.M. Wilson, A.E. Goodship, The effect of exercise-induced localised hyperthermia on tendon cell survival, J. Exp. Biol. 200 (1997) 1703–1708.
[36] N.L. Millar, J.H. Reilly, S.C. Kerr, A.L. Campbell, K.J. Little, W.J. Leach, B.P. Rooney, et al., Hypoxia: a critical regulator of early human tendinopathy, Ann. Rheum. Dis. 71 (2012) 302–310.
[37] J.I. Liang, P.C. Lin, M.Y. Chen, T.H. Hsieh, J.J. Chen, M.L. Yeh, The effect of tenocyte/hyaluronic acid therapy on the early recovery of healing Achilles tendon in rats, J. Mater Sci. Mater Med. 25 (2014) 217–227.
[38] A. Wang, W. Breidahl, K.E. Mackie, Z. Lin, A. Qin, J. Chen, M.H. Zheng, Autologous tenocyte injection for the treatment of severe, chronic resistant lateral epicondylitis: a pilot study, Am. J. Sports Med. 41 (2013) 2925–2932.
[39] J. Chen, Q. Yu, B. Wu, Z. Lin, N.J. Pavlos, J. Xu, H. Ouyang, et al., Autologous tenocyte therapy for experimental Achilles tendinopathy in a rabbit model, Tissue Eng. Part A 17 (2011) 2037–2048.
[40] C. Stoll, T. John, C. Conrad, A. Lohan, S. Hondke, W. Ertel, C. Kaps, et al., Healing parameters in a rabbit partial tendon defect following tenocyte/biomaterial implantation, Biomaterials 32 (2011) 4806–4815.
[41] C. Stoll, T. John, M. Endres, C. Rosen, C. Kaps, B. Kohl, M. Sittinger, et al., Extracellular matrix expression of human tenocytes in three-dimensional air-liquid and PLGA cultures compared with tendon tissue: implications for tendon tissue engineering, J. Orthop. Res. 28 (2010) 1170–1177.
[42] A.D. Mazzocca, M.B. McCarthy, D. Chowaniec, M.P. Cote, C.H. Judson, J. Apostolakos, O. Solovyova, et al., Bone marrow-derived mesenchymal stem cells obtained during arthroscopic rotator cuff repair surgery show potential for tendon cell differentiation after treatment with insulin, Arthroscopy 27 (2011) 1459–1471.
[43] J.E. Russell, P.R. Manske, Ascorbic acid requirement for optimal flexor tendon repair in vitro, J. Orthop. Res. 9 (1991) 714–719.
[44] B. de Crombrugghe, V. Lefebvre, R.R. Behringer, W. Bi, S. Murakami, W. Huang, Transcriptional mechanisms of chondrocyte differentiation, Matrix Biol. 19 (2000) 389–394.
[45] A. Takimoto, M. Oro, Y. Hiraki, C. Shukunami, Direct conversion of tenocytes into chondrocytes by Sox9, Exp. Cell Res. 318 (2012) 1492–1507.
[46] T. Barsby, D. Guest, Transforming growth factor beta3 promotes tendon differentiation of equine embryo-derived stem cells, Tissue Eng. Part A 19 (2013) 2156–2165.

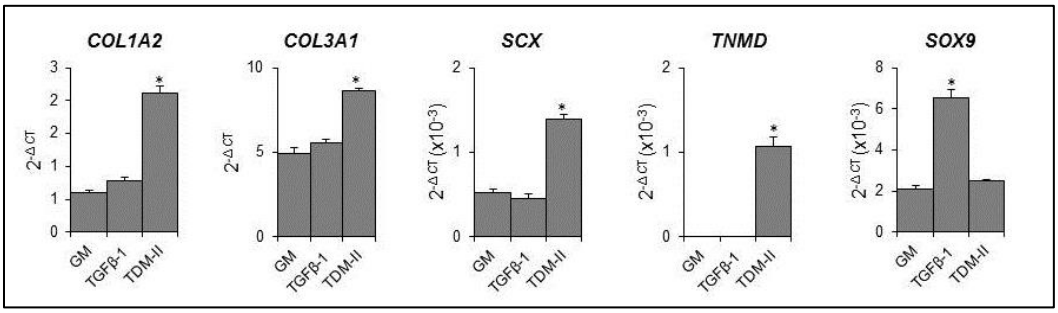
Supplementary Figures



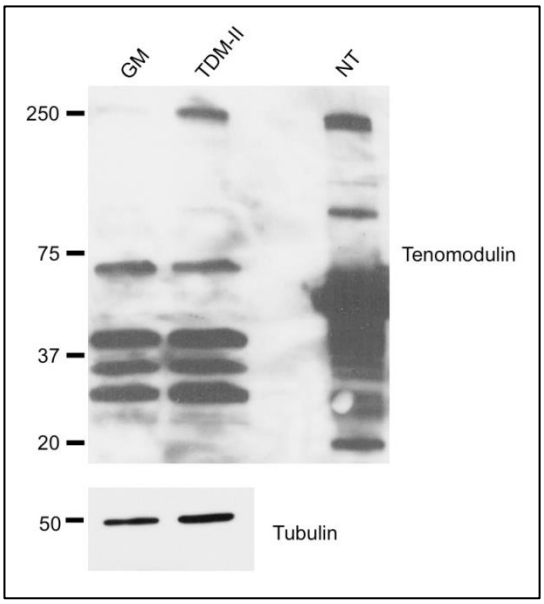
Supplementary Fig. 1: Representative images of equine ASCs (A and B) and equine tenocytes (C and D) stained for Alizarin red (A and C) or Oil Red O (B and D) at day 14 after incubation in osteogenic or adipogenic differentiation medium respectively. Controls refer to cultures incubated in growth medium only. Scale bar = 3.9 mm (A, C), 100 μm (B, D).



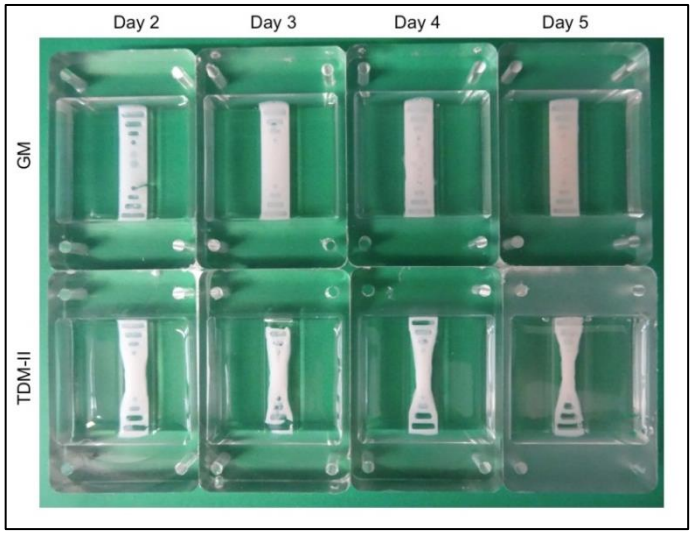
Supplementary Fig. 2: Representative high magnification images of hematoxylin and eosin stained paraffin wax sections of equine TC-microtissue spheroids cultured for 4 days (A) and 6 days (B) in GM. *Arrows*, identification of elongated tenocytes within microtissue spheroid at day 6. Scale bar = 25 μm.



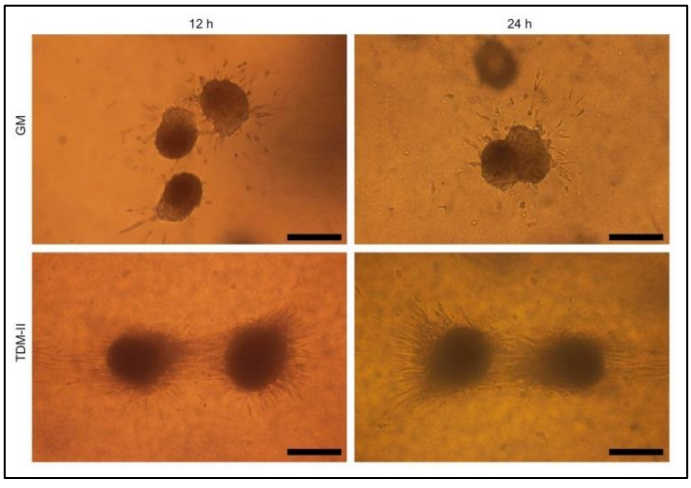
Supplementary Fig. 3: qRT-PCR analysis of *COL1A2*, *COL3A1*, *SCX*, *TNMD* and *SOX9* in equine tenocytes cultured in 3D microtissue spheroids for 6 days. In each case, gene expression in equine tenocytes cultured in growth medium (GM) alone was compared to cells cultured in either TGFβ-1 alone (TGFβ-1) or tenogenic differentiation medium II (TDM-II). Data was normalized to *GAPDH* and is presented as $2^{-\Delta CT}$. * $p < 0.01$ as compared to tenocytes cultured in GM. Significance was determined by one-way ANOVA and Tukey's post-hoc test. (± S.D. triplicates).



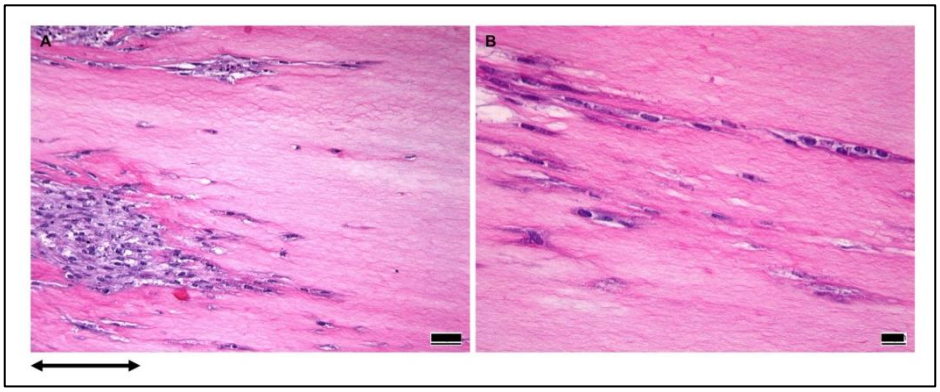
Supplementary Fig. 4: Western blot analysis of protein lysates (40 μg) from equine TC-microtissues treated with GM (GM) or TDM-II (TDM-II). Protein (10 μg) from native equine tendon (NT) was used as positive control. Protein samples were loaded onto an SDS-PAGE gel and tenomodulin detected using a specific antibody targeting the N-terminus of human tenomodulin. A monoclonal tubulin antibody was used as a loading control.



Supplementary Fig. 5: Macroscopic images of collagen gel constructs anchored in silicon molds at 2, 3, 4 and 5 days after TC-microtissue seeding of GM or TDM-II treated cultures. Scale bar = 1 cm.



Supplementary Fig. 6: Representative images of collagen gel-embedded GM- and TDM-II-treated TC-microtissues in situ at 12 and 24 h after seeding. Scale bar = 100 μm.



Supplementary Fig. 7: Visualization of tenocyte and collagen fibre alignment in hematoxylin and eosin stained paraffin wax sections of 3D collagen constructs 5 days after seeding with TDM-II-treated TC-microtissues. (A) Scale bar = 50 µm. (B) Scale bar = 20 µm. Arrow, orientation of collagen gels based on their positioning in anchored silicon molds.

5.2 Additional / unpublished data

5.2.1 Characterization of equine tenocytes in the MT format

Gravity assisted assembly of equine adult tenocytes (EATs) and fetal tenocytes (EFTs) leads to the formation of MTs. Size of MTs was dependent on cell number per tissue and incubation time, where a decrease in MT size was observed over time (Fig. 5, 6). Culture periods of 6 days were feasible with 25 µl of medium per well in the Terasaki plates for hanging drop cultures.

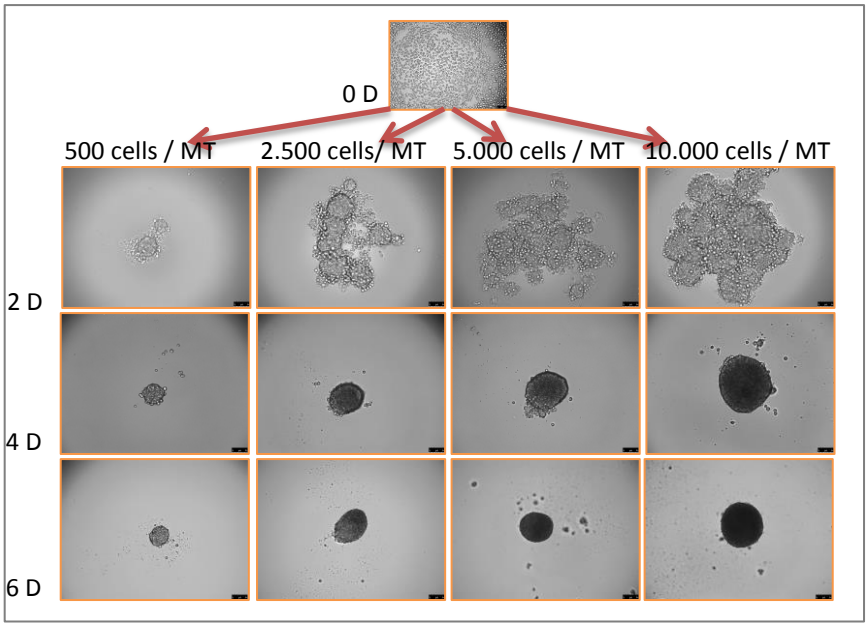


Fig. 5: Formation of MTs with different numbers of EATCs/MT

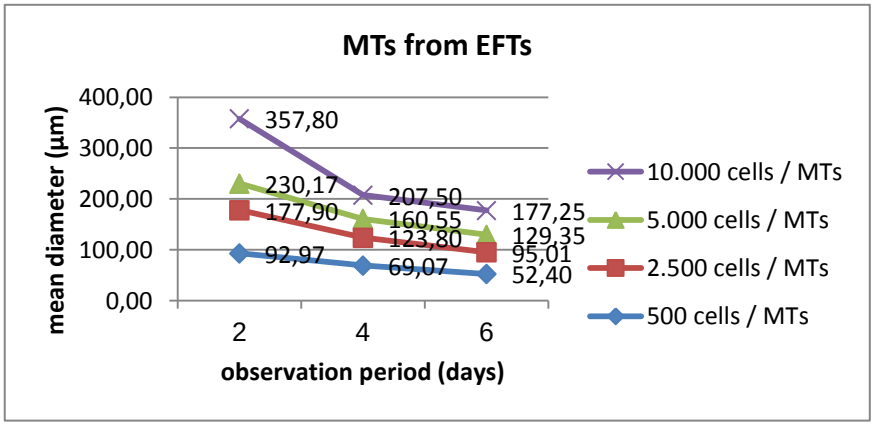


Fig. 6: Decrease in size over time in MTs from EFTs

Incomplete assembly of the cells was observed at 2 days in hanging drop culture as identified by the irregular shape of MTs in hematoxylin and eosin (H&E) stained paraffin wax sections (Fig. 7A and 8A). After 4 days in hanging drops, the cells had fully assembled to form round MTs (Fig. 7B and 8B). By

day 6, elongated cells were visible in the MTs (Fig. 7C). However, in MTs assembled from EFTs, small dark stained cell fragments were visible at 4 days (Fig. 8C). Cell fragmentation and the resulting empty spaces within the MTs composed of EFTCs was even more obvious at day 6. Active caspase immunostaining revealed increasing rates of apoptosis over time in MTs composed of EFTs (Fig. 8D) as compared to EATs MTs (Fig. 7D).

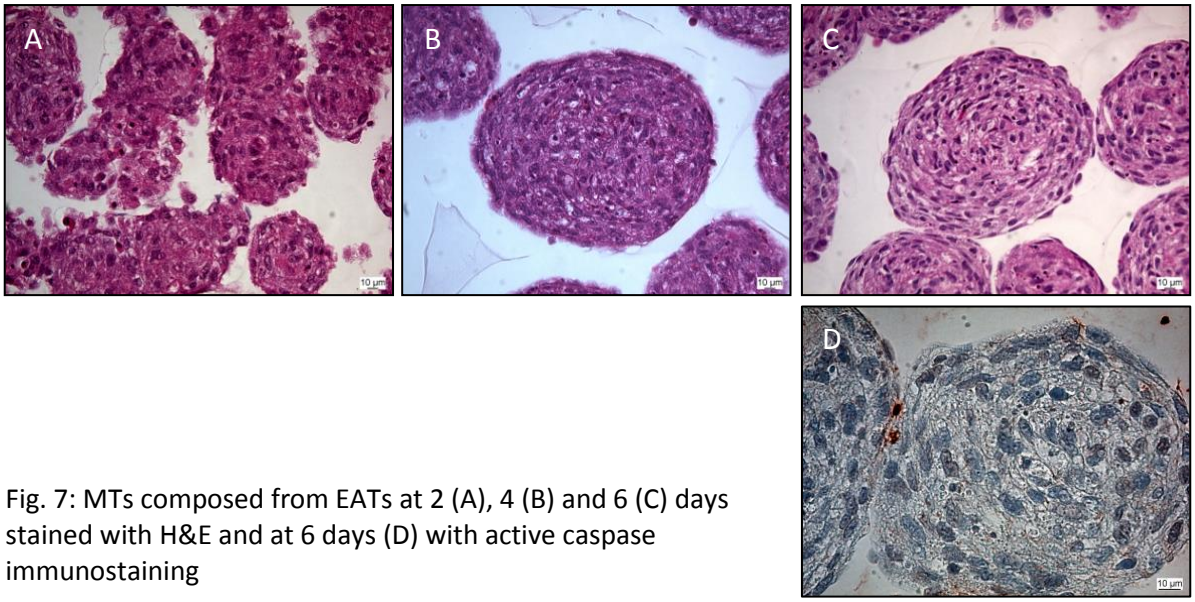


Fig. 7: MTs composed from EATs at 2 (A), 4 (B) and 6 (C) days stained with H&E and at 6 days (D) with active caspase immunostaining

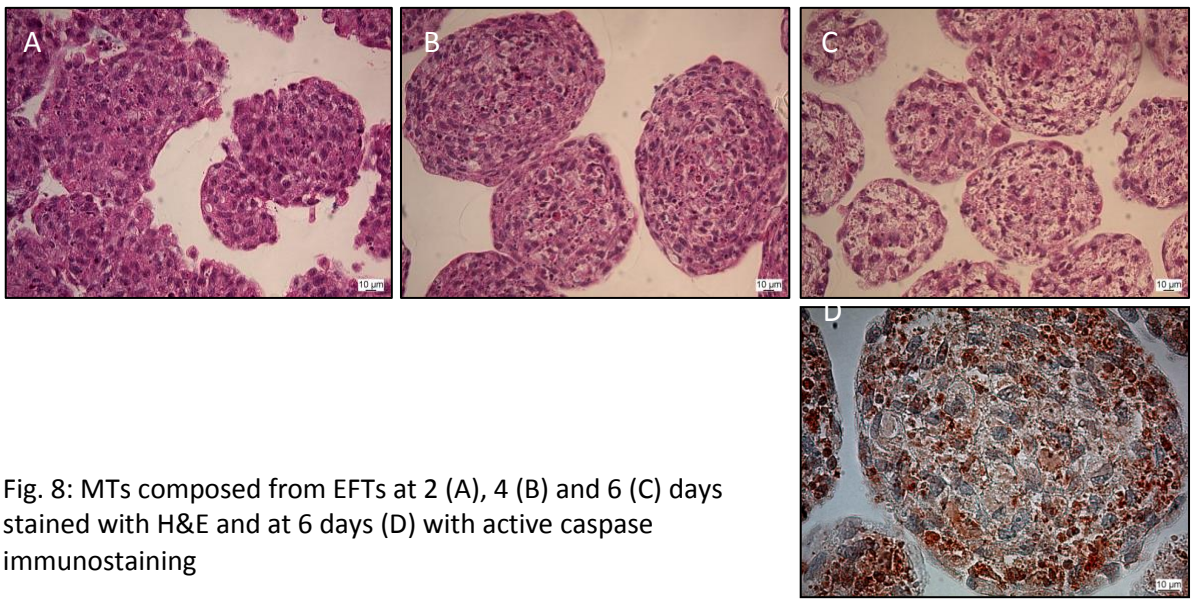


Fig. 8: MTs composed from EFTs at 2 (A), 4 (B) and 6 (C) days stained with H&E and at 6 days (D) with active caspase immunostaining

To analyze gene expression, RT-PCR and RT-qPCR was carried out. For RT-PCR, primers were designed for *COL1A2*, *TNC*, *SCX*, *TNMD* and the ribosomal subunit *18s*, which served as the housekeeping gene control (Fig. 9). In native tendon all selected genes were expressed (Fig. 10). In contrast to this *TNMD* was not expressed in monolayer culture or in MTs composed of EATCs (Fig. 11) and EFTCs (Fig. 12).

Target Gene	Product size	Annealing temp.	Sequence
COL1A2	152 bp	55° C	F: 5’ GAGCAGCGGTTACTACTGGATT 3’ R: 5’ TCTTGGCCTTGGAACTTCTG 3’
TNC	177 bp	55° C	F: 5’ TGGCCACTTACTTACCTGCAC 3’ R: 5’ TGGTCTCTGGCCTCCTTAGA 3’
SCX	200 bp	55° C	F: 5’ CTGGCCTCCAGCTACATCTC 3‘ R: 5‘ CTGAGGCAGAAGGTGCAGAT 3’
TNMD	203 bp	55° C	F: 5‘ GATCTTCACTTCCCTACCAACG 3‘ R: 5‘ CCTCGACGGCAGTAAATACAA 3‘
18S	219 bp	55° C	F: 5’ CGCGGTTCTATTTTGTTGGT 3’ R: 5’ AGTCGGCATCGTTTATGGTC 3’

Fig. 9: primer sequence of target genes for RT-PCR

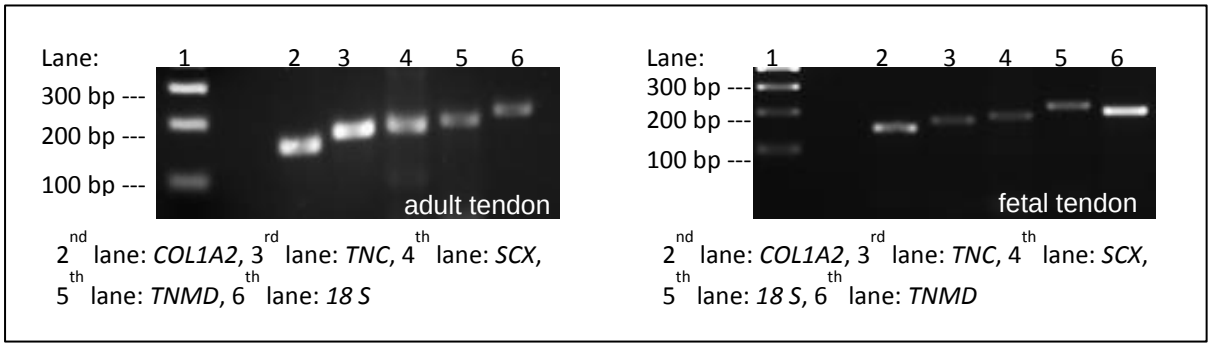


Fig. 10: RT-PCR from native adult and fetal tendon

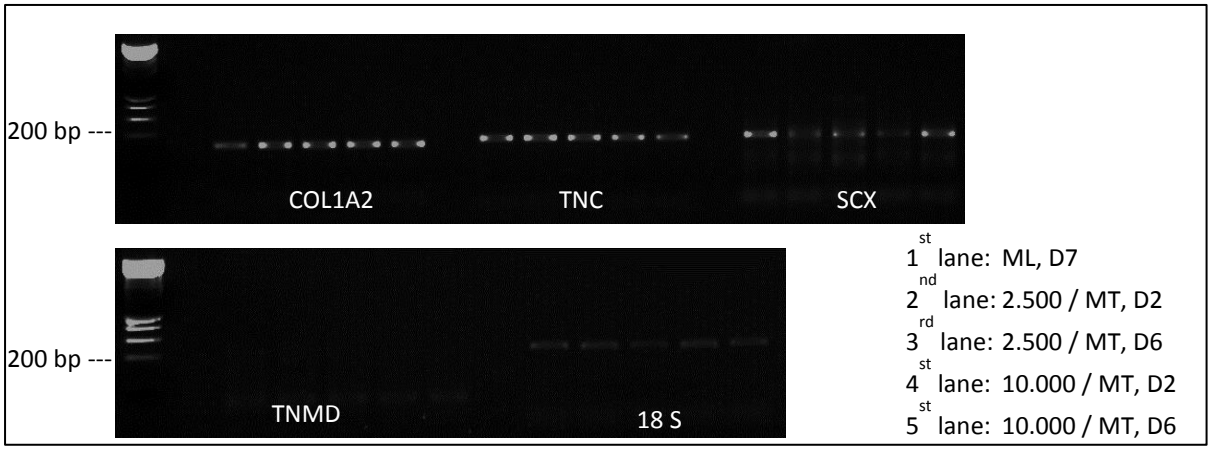


Fig. 11: RT-PCR from EATs in monolayer culture and in MTs

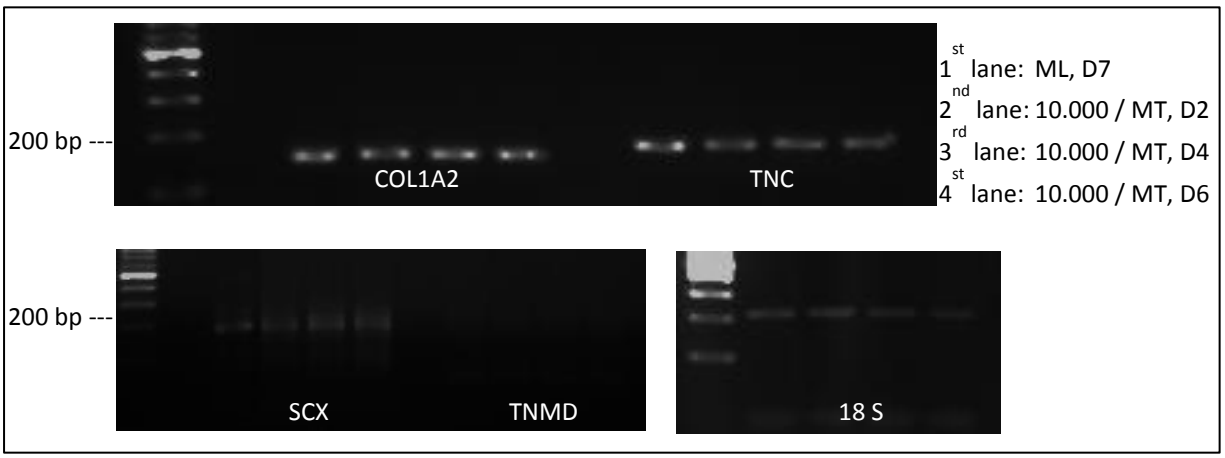


Fig. 12: RT-PCR from EFTs in monolayer culture and in MTs

TaqMan gene expression assays for equine *COL1A2*, *SCX*, *TNMD*, *COL2A1*, *SOX9* and *GAPDH* were commercially available and therefore used for RT-qPCR. In native tissue samples expression levels of *COL1A2* and *TNMD* were significantly higher in fetal tendon compared to adult tendon, whereas *SCX* expression was higher in adult tendon tissue (Fig. 13).

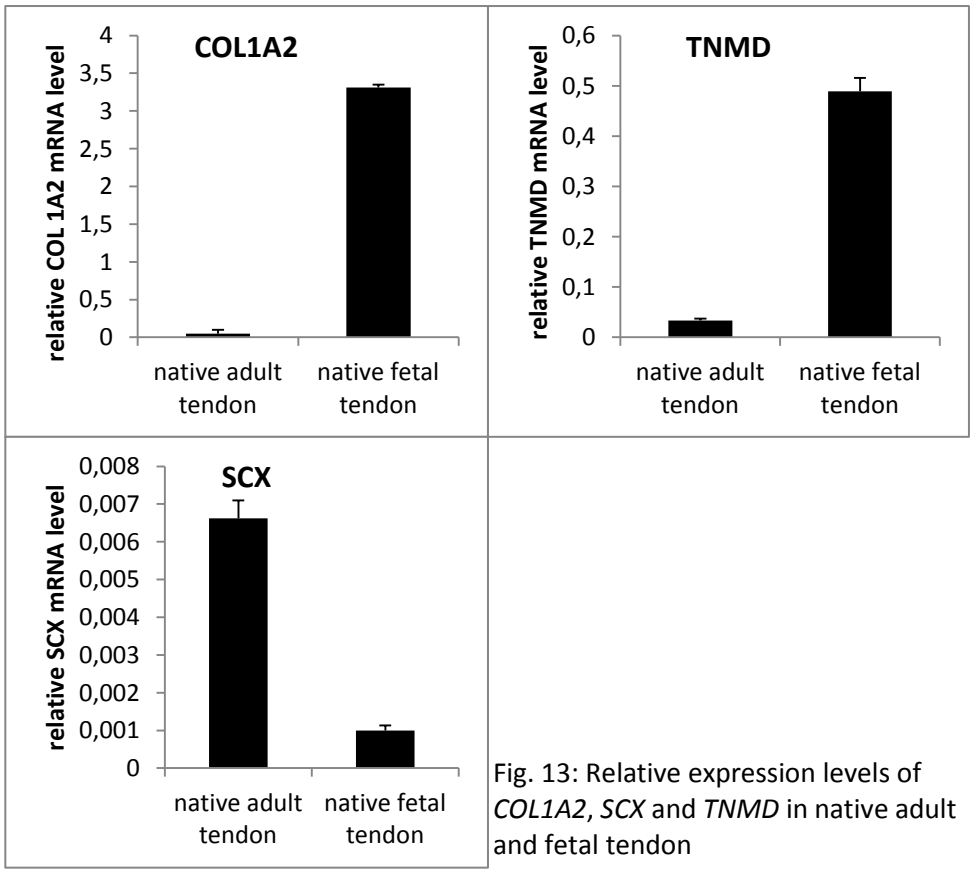


Fig. 13: Relative expression levels of *COL1A2*, *SCX* and *TNMD* in native adult and fetal tendon

In monolayer culture, *COL1A2* expression levels were comparable between EATs and EFTs, although *SCX* expression levels were lower in EFTs (Fig. 14). *COL1A2* expression levels were lower in the MT format as compared to monolayer culture, and decreased over time. *SCX* expression levels were comparable between EAT-MTs and monolayer cultures (Fig. 14). In MT-EFTs cultures, expression levels for *COL1A2* and *SCX* were already much lower after 2 days and rapidly decreased over time, corresponding to the increased rate of apoptosis seen in histology sections. *TNMD* expression was not detectable in any of the culture formats at any time point (data not shown).

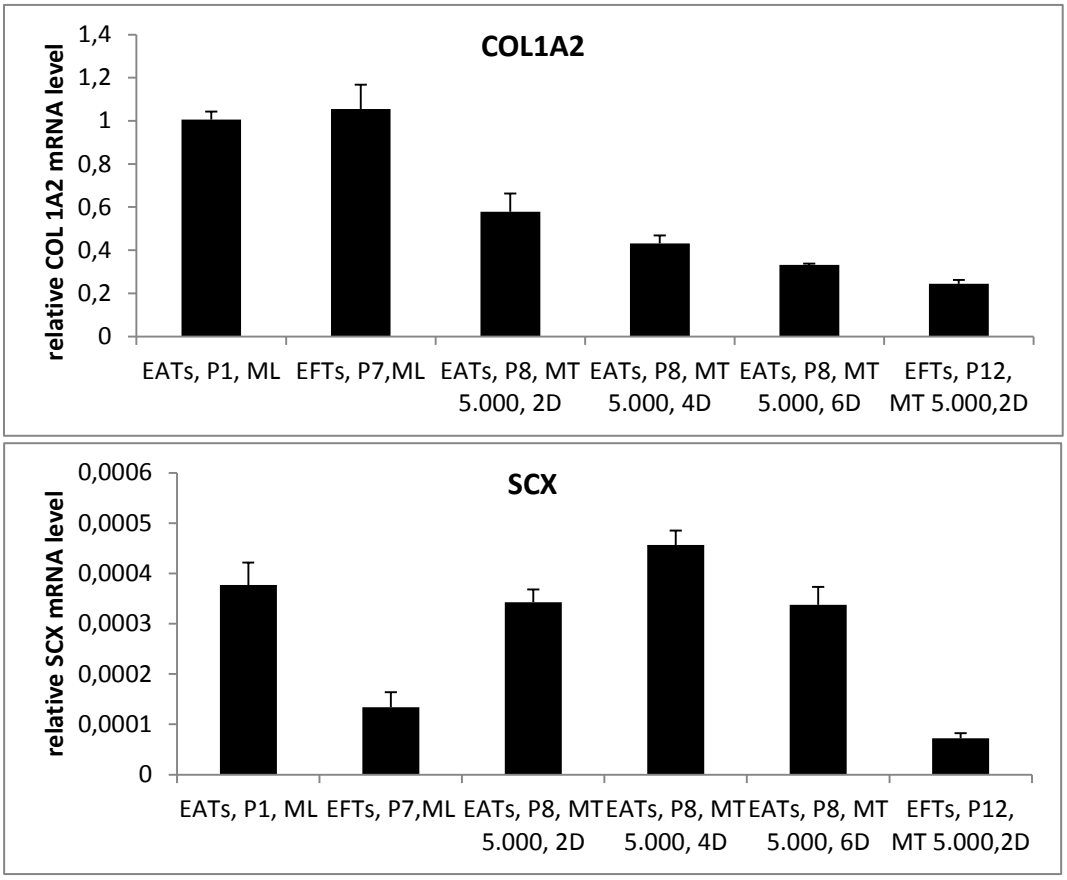


Fig. 14: Relastive expression levels of *COL1A2* and *SCX* in monolayer culture and MTs from EATs and EFTs

To evaluate a possible protective effect of dexamethasone on tenocyte viability in 3-D culture, different concentrations of dexamethasone were added to the medium in the hanging drop system. A concentration dependent effect on time for MT formation could be seen in EFTs (Fig. 15). Unfortunately, gene expression levels of *COL1A2* and *SCX* were further decreased (Fig. 16). Because of this EFTs were considered as being an unsuitable cell source for the MT format.

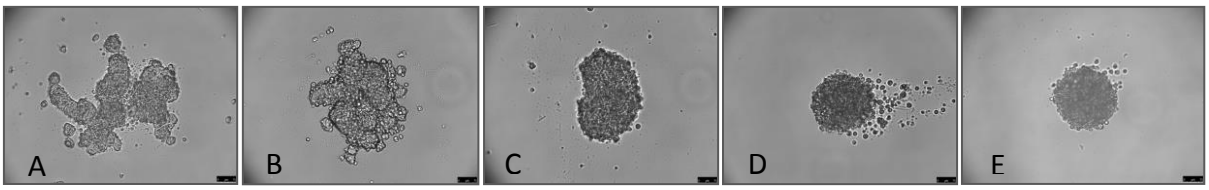


Fig. 15: Dexamethasone concentration dependent accelerated formation of MTs from EFTs; (A) 0µM, (B) 1 µM, (C) 10 µM, (D) 100 µM, (E) 1000 µM Dexamethasone, observation period 2 days

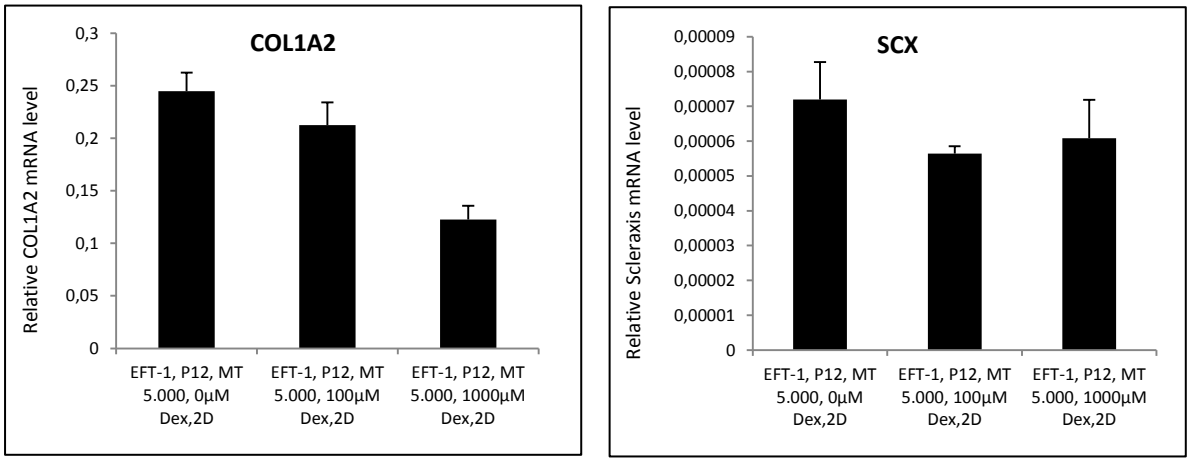


Fig. 16: Relative expression levels of *COL1A2* and *SCX* in MTs composed of EFTs under the influence of different concentrations of dexamethasone.

5.2.2 Co-culture of EATs and EFTs with MSCs

Equine MSCs were harvested from bone marrow or adipose tissue and cultured in the MT format alone (Fig. 17), or in combination with EATs or EFTs (Fig. 18). Irrespective of cell ratio, assembly of MTs was comparable to hanging drops consisting of EATs or EFTs alone. Interestingly, the expected apoptotic appearance of EFTs in MTs was not observed when co-cultured with MSCs, suggesting a possible protective effect of MSCs on EFTs survival. However, further evaluation is needed to confirm this.

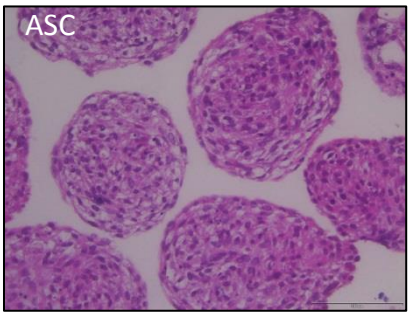


Fig. 17: H&E stained paraffin wax section of MTs composed of equine ASC

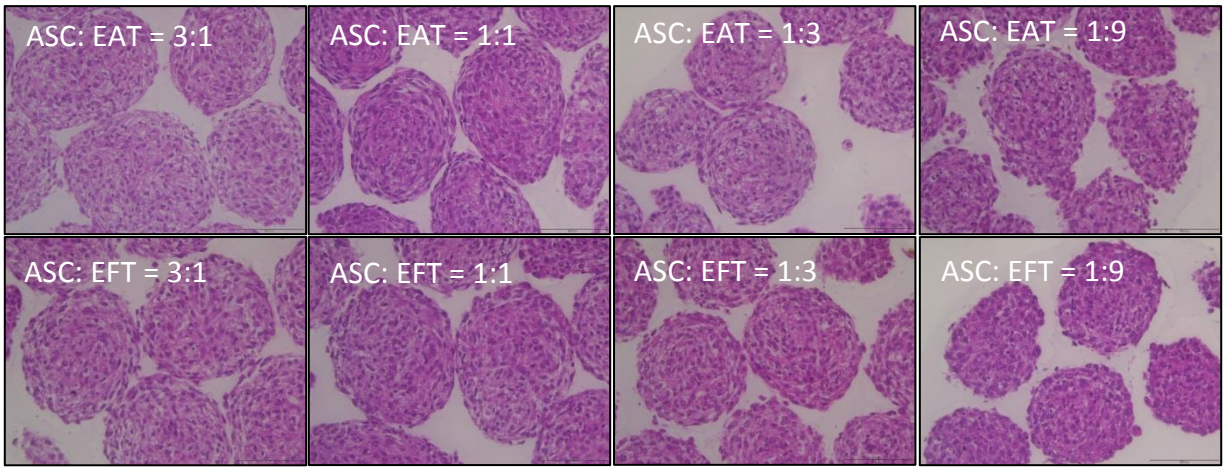


Fig. 18: H&E stained paraffin wax sections of MTs composed of co-culture of ASCs and EATs or EFTs

Osteogenic differentiation capacity of MSCs was evaluated in the MT format. Interestingly osteogenic differentiation, demonstrated by positive Alizarin Red S staining was accelerated in the MT format as compared to monolayer culture (Fig. 19) and was faster in BMSCs compared to ASCs (data not shown). RT-qPCR analysis was performed for *COL1A2*, *COL2A1*, *COL3A1*, *SCX*, *TNMD*, *TNC*, *SOX9*, *TIMP1*, *MMP1*, *MMP3*, *MMP13*, *IGF1* and *IL1* using custom designed primers. Relative gene expression levels are shown in Fig. 20 and 21. *COL2A1*, *TNMD* and *IL1* were not detectable in any of the cultures (data not shown).

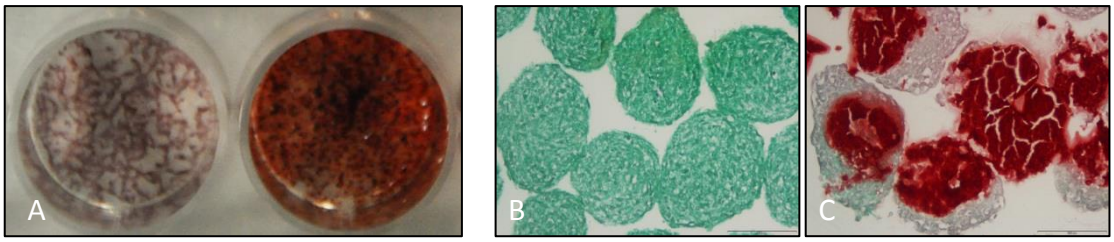
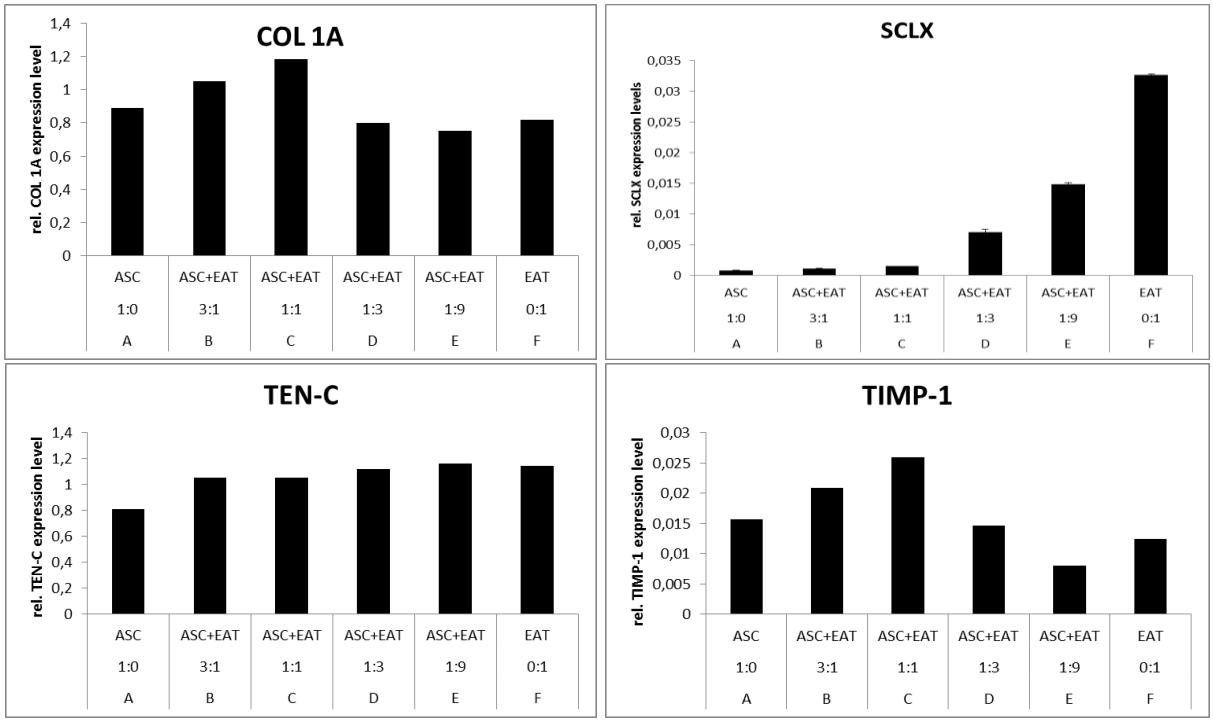


Fig. 19: A: monolayer culture of equine ASCs with normal growth medium (left panel) or osteoinduction medium (right panel) at day 14; B-C: ASC-MTs cultured for 6 days without osteoinduction (B) or with osteoinduction (C). In all cases, red staining signifies mineral deposition; Alizarin Red S staining

When calculated as a ratio, co-culture of EATs and ASCs demonstrated a decrease in *COL3A1:COL1A2* (Fig. 22). Evaluating the influence of ASCs on equine tenocytes in the MT format by fold change of gene expression levels was somewhat disappointing. Tendon associated genes like *SCX* and *TNC* were lower in the co-cultures. The slight increase in *COL1A2* expression in the co-culture may be explained by the fact, that expression of type I collagen is higher in ASCs than in EATs (Fig. 23).



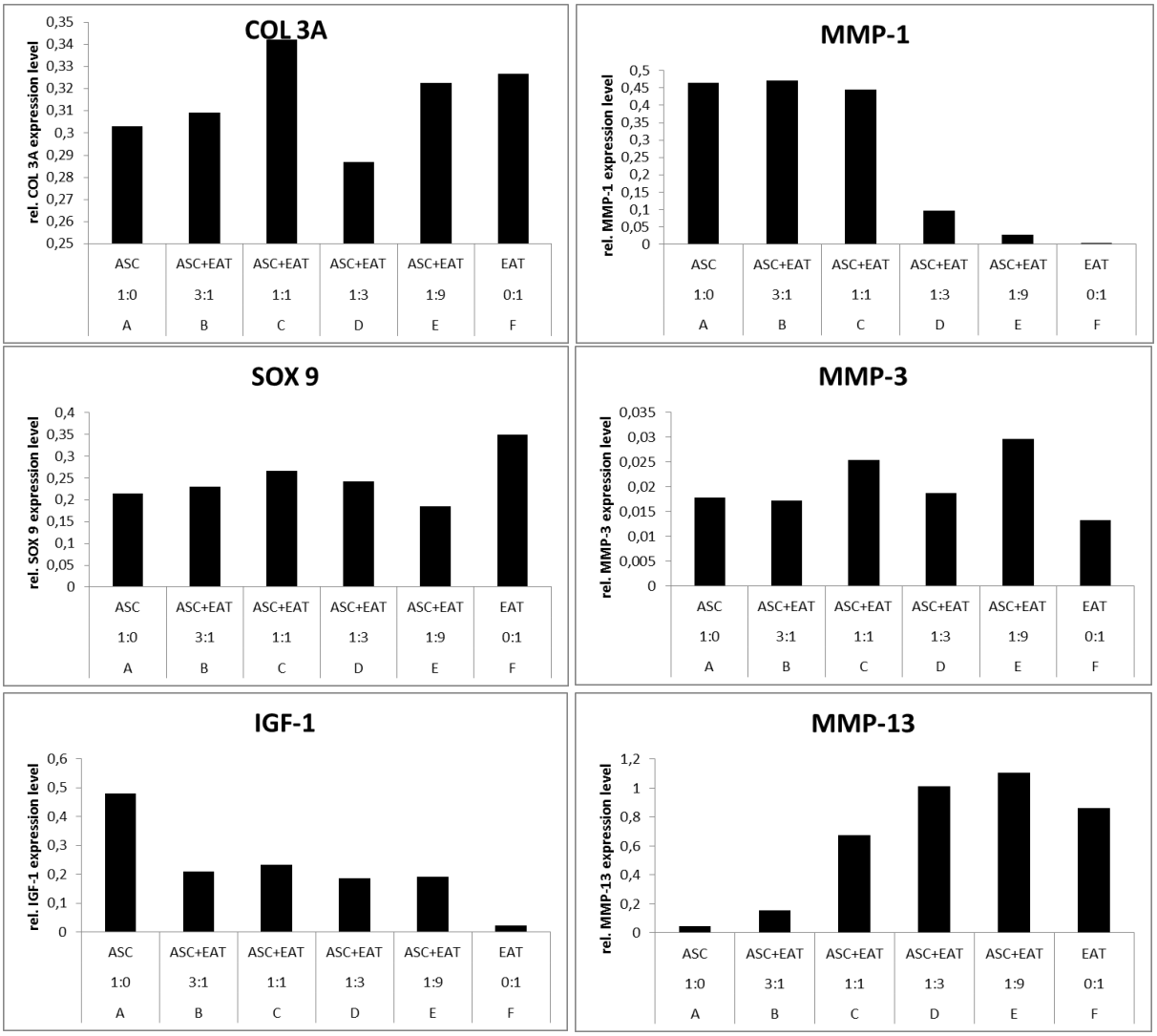
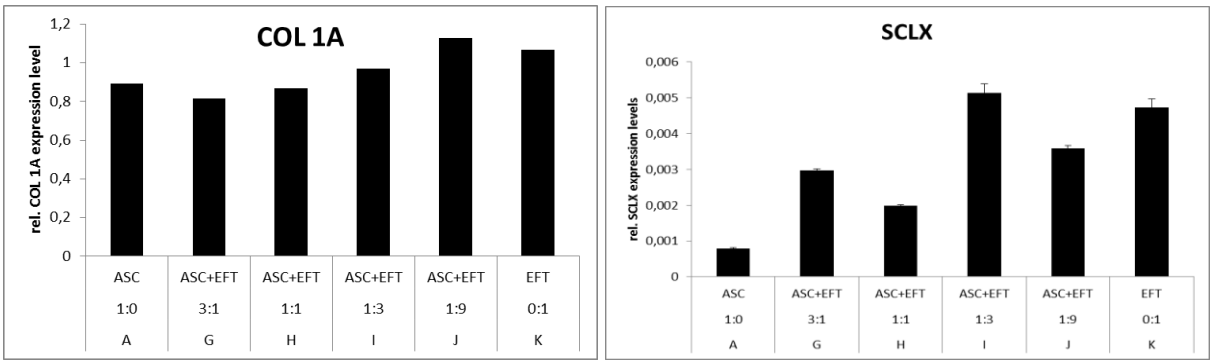


Fig. 20: relative gene expression levels of different ratios of co-culture of EATs and ASCs in MTs at day 6, *GAPDH* served as house keeping gene



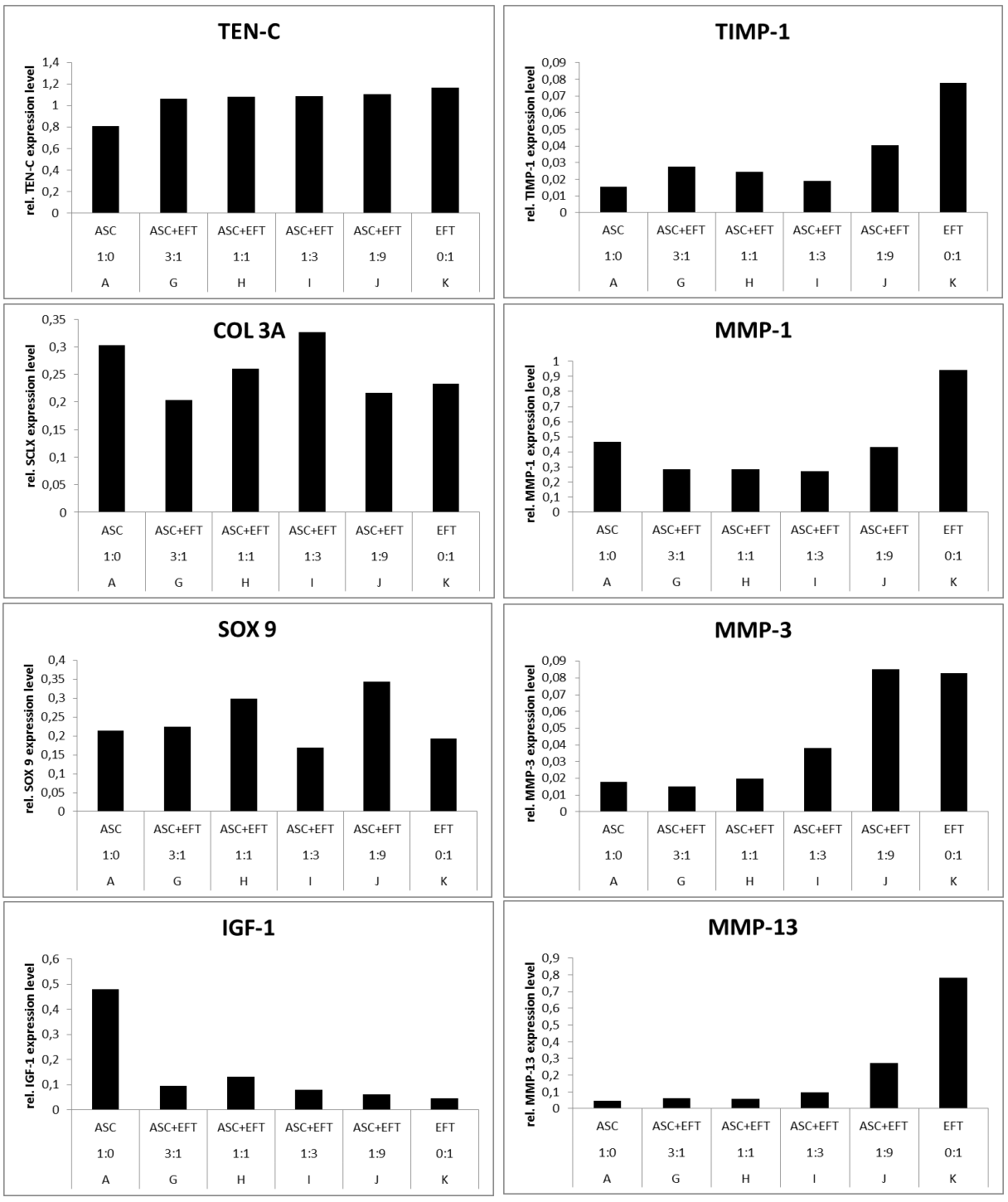


Fig. 21: relative gene expression levels of different ratios of co-culture of EFTs and ASCs in MTs at day 6, *GAPDH* served as house keeping gene

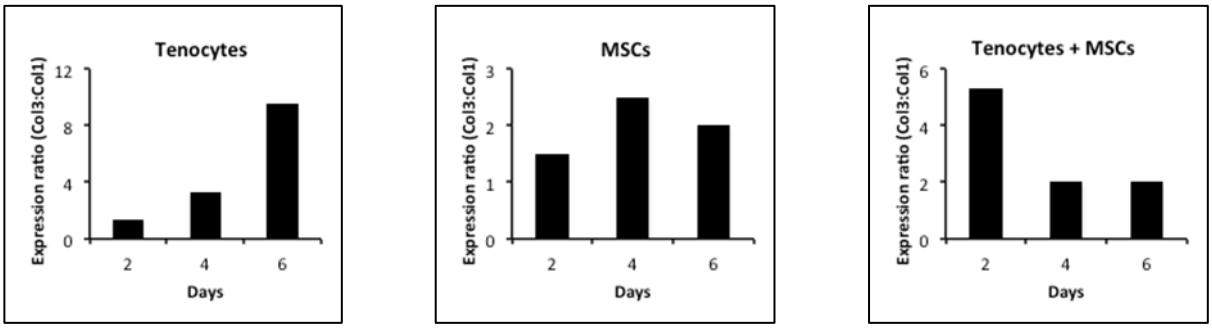


Fig. 22: Ratio of *COL3A:COL1A* relative expression in 3-D co-culture of EATs, ASCs, or EATs + ASCs; expression levels were normalized to the housekeeping gene *GAPDH* and expression values expressed as ratios of *COL3A:COL1A*.

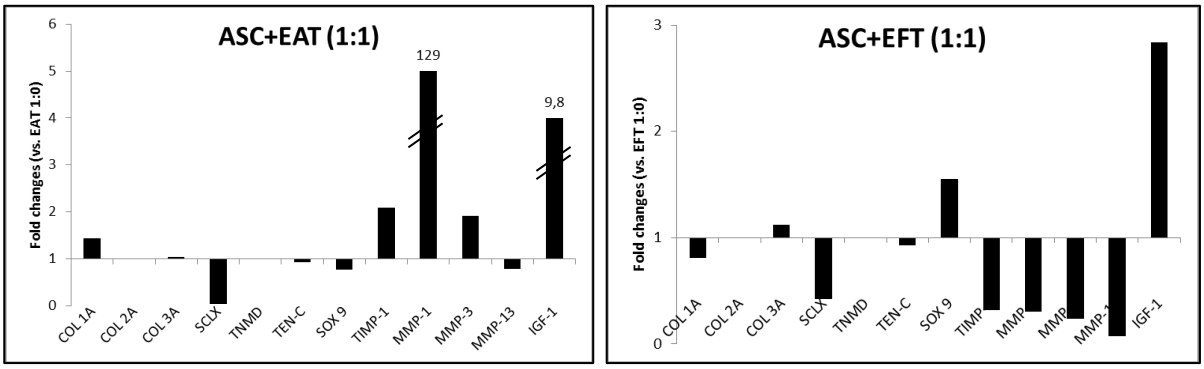


Fig. 23: Fold change of gene expression levels comparing MTs of EATs or EFTs alone and in combination with ASCs at day 6

5.2.3 Mechanical stimulation of equine tenocytes

Mechanical stimulation was initially performed using a custom made bioreactor, which had previously been used to generate tissue-engineered blood vessels from human artery-derived fibroblasts and endothelial cells (346). The bioreactor setup contained a control unit, a pulsatile pump, a medium reservoir and the assembly device within a falcon tube (Fig. 24). The assembly device was modified in a way that MTs could be assembled in a newly designed agarose mold around the silicon tube (Fig. 25). To reduce the risk of infection and possible loss of pressure, tubing was replaced by less elastic plastic tubes using a Luer-Lock system. Furthermore, new media reservoirs with a Luer-Lock system were produced. MTs were harvested and transferred into the agarose mold and fused to form a ring shaped tissue construct (Fig. 26).

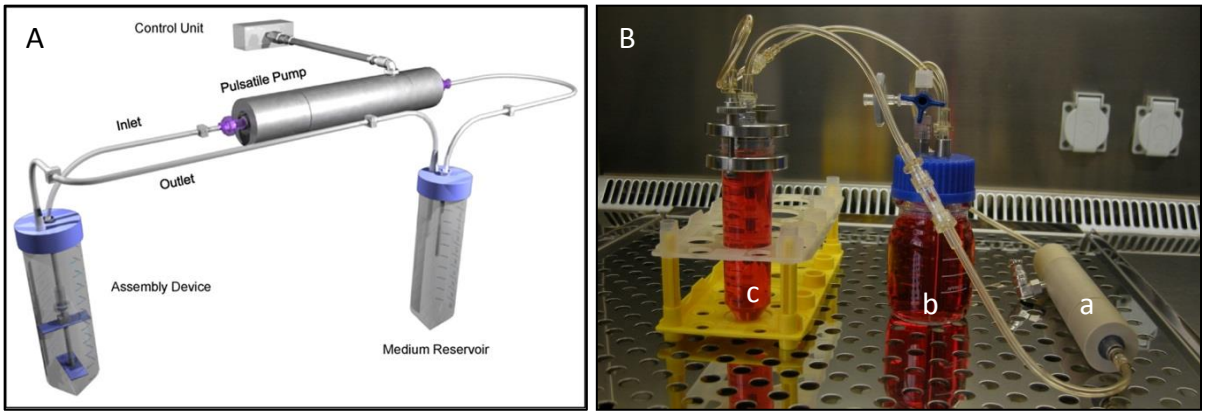


Fig. 24: A: schematic drawing of the custom made bioreactor setup (346); B: image of the modified system with a) pulsatile pump, b) medium reservoir and c) assembly device

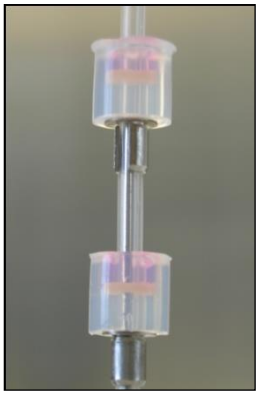


Fig. 25: agarose mold to enable assembly of MTs around the silicon tube

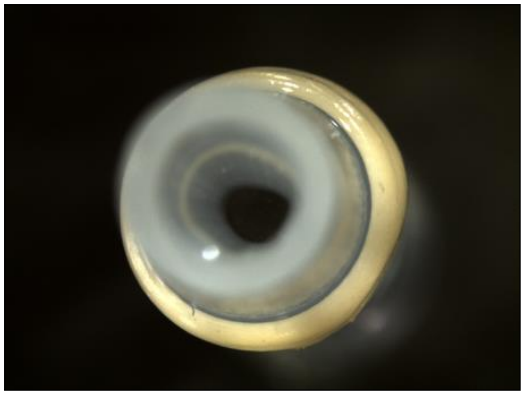


Fig.: 26: macroscopic view of a ring shaped tendon tissue construct from non-stimulated EFTs

As histologic evaluation revealed no obvious differences between mechanically stimulated and unstimulated constructs (Fig. 27), expansion of the silicon tube and thereby the amount of mechanical stimulation was assessed by means of a high resolution slow motion camera and revealed only minor expansion rates. Reducing the inner lumen of the silicon tube with a custom made reduction device to an inner diameter of 5 mm, led to controllable expansion rates from 0.7 to 7.2%. Stimulation with 5% expansion of the silicon tube resulted in ring shaped tissue constructs, which in contrast to unstimulated constructs displayed a rough and irregular surface (Fig. 28). Active caspase immunostaining revealed lower rates of apoptosis in mechanically stimulated as compared to unstimulated constructs (Fig. 29). As further histologic evaluation detected no influence of mechanical stimulation on cell orientation (Fig. 29) and gene expression analysis (Fig. 30) was somewhat uninformative, this custom made, scaffold free bioreactor was not continued any further.

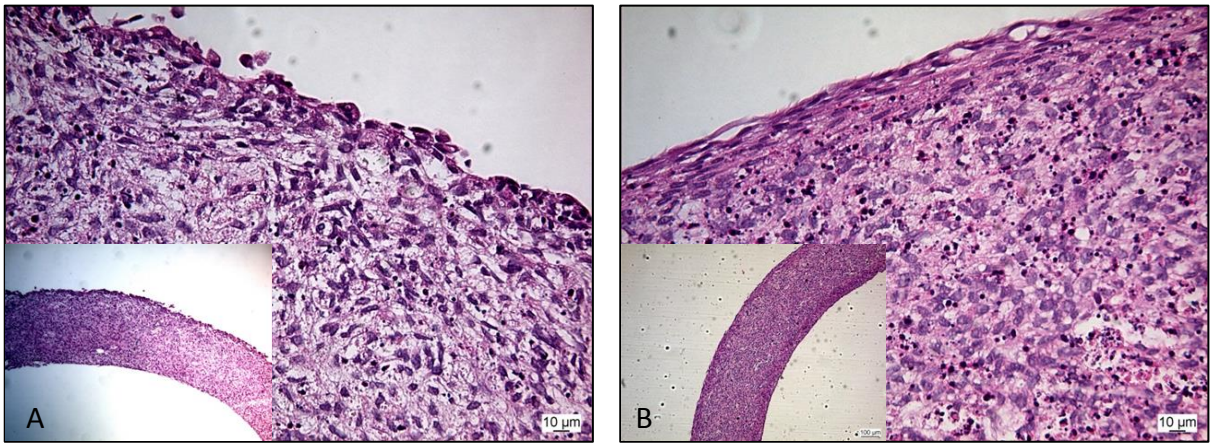


Fig. 27: H&E stained paraffin wax section of ring shaped tendon tissue construct composed from MTs of EFTs cultivated without (A) or with mechanical stimulation (B) for 7 days

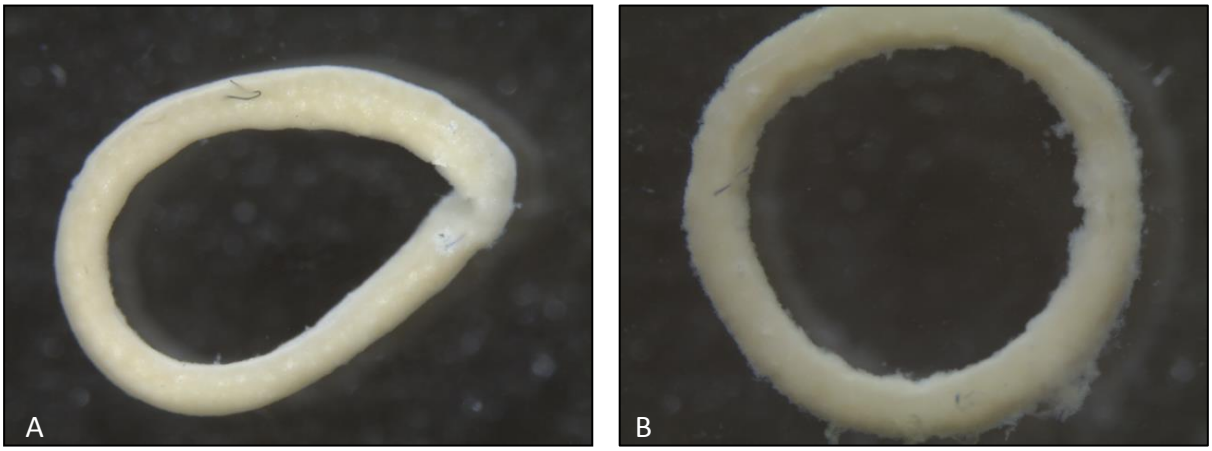


Fig. 28: ring shaped tissue constructs from EATs; (A) was cultivated for 10 days without mechanical stimulation, (B) was cultivated unde 5% mechanical stimulation for 10 days

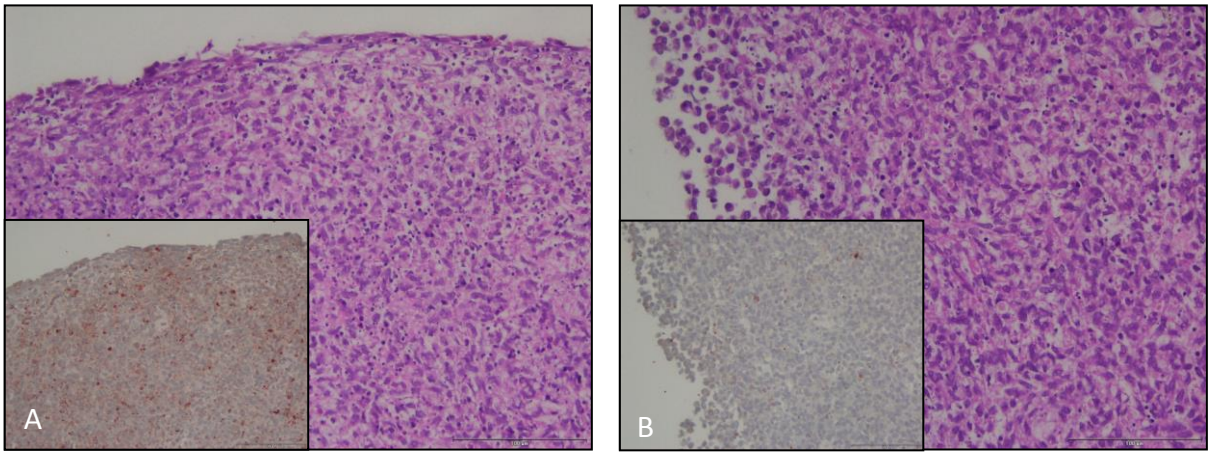


Fig. 29: H&E stained paraffin wax section of ring shaped tissue constructs composed of EATs; construct (A) has been cultivated without and construct (B) with 5% mechanical stimulation for 10 days. Inlet shows active caspase 3 immunostaining of the ring shaped tissue constructs

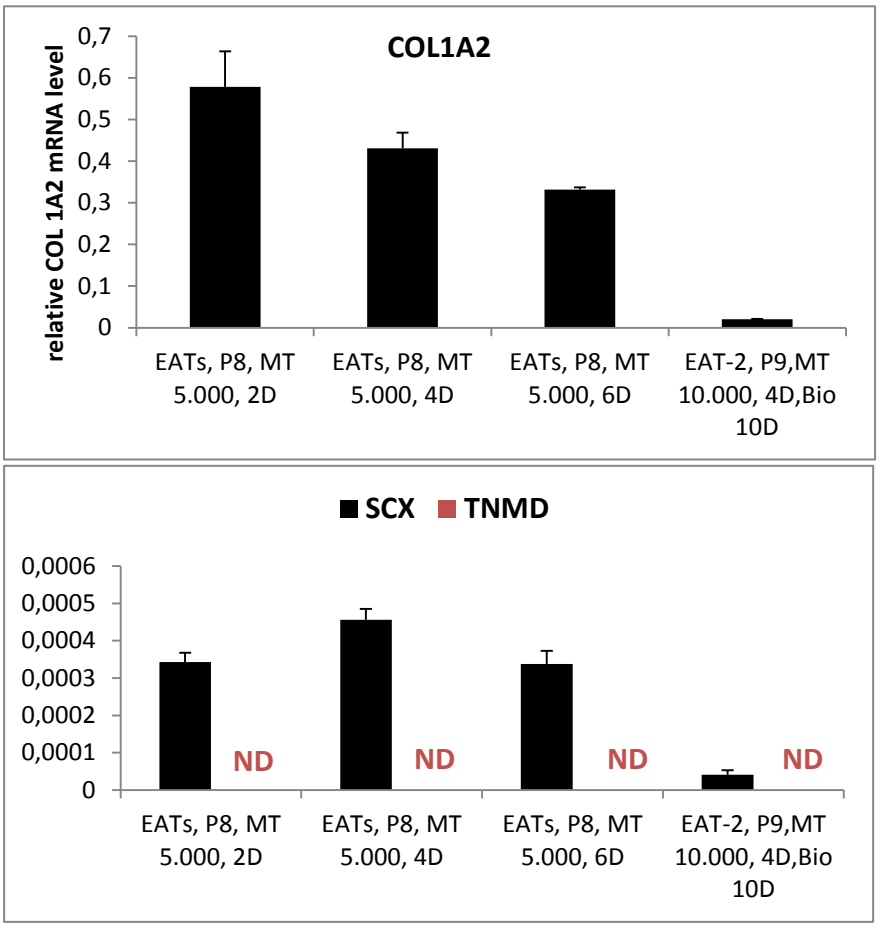


Fig. 30: relative expression levels of *COL1A2*, *SCX* and *TNMD* in MTs composed of EATs over time and in a ring shaped tissue construct after 10 days of mechanical stimulation; ND: not detectable

To further characterize MTs under the influence of mechanical stimulation, a pilot study was carried out using the STREX-ST-140™ from B-Bridge International in cooperation with the Institute for Biomechanics, Department of Health Sciences and Technology at the ETH Zurich. The STREX-ST-140™ allows for custom set different uniaxial strain rates at different frequencies. As commercially available chambers allow only for 2-D culture, molds to produce silicon chambers for 3-D culture were designed in collaboration with the ETH, Zurich. Single EATs and MTs composed of EATs were embedded in a 0.8% collagen scaffold and, after a setting time of 2 days, stimulated with 5% strain at 1 Hz twice a day for 60 minutes each for a total period of 12 days. In single cells, no differences between the stimulated group and the unstimulated controls were detected. Cells exhibited a round shape with no orientation to the direction of mechanical stretching. Overall, cell density was low. There were no signs for remodeling of the extracellular collagen matrix visible (Fig. 31). In MTs cultivated in the collagen matrix for 12 days without mechanical stimulation, empty spaces, initially occupied by MTs, and low numbers of rounded cells in the scaffold were observed (Fig. 32). In contrast, in MTs cultivated under mechanical stimulation, elongated fibroblast-like cells orientated according to the direction of mechanical stimulation could be identified. Furthermore, cells with a tenocyte like phenotype migrating out of the MTs and a first staggered arrangement of cells was visible. The collagen matrix appeared less homogeneous as compared to the other groups with fiber orientation according to the direction of strain applied (Fig. 32). With the question in mind, if a collagen scaffold is necessary for mechanical stimulation of tendon-derived fibroblasts *in vitro* or if the old bioreactor was unsuitable because of other reasons, a ring shaped tissue construct, assembled in the agarose mold, was cut and placed strait in the collagen scaffold. For unknown reasons, stimulation in the STREX-ST-140™ had no detectable effect, not at the interface between the collagen scaffold and the MTs, nor within the tissue construct itself (Fig. 33).

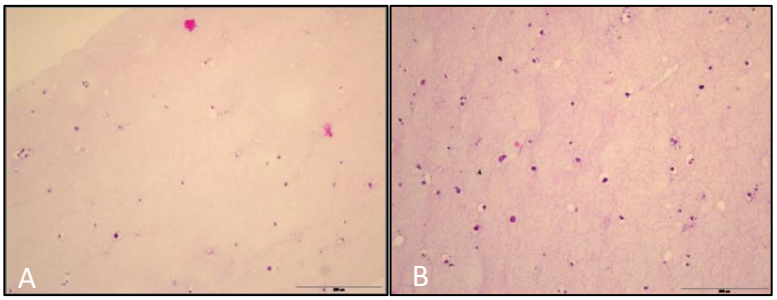


Fig. 31: H&E stained paraffin wax section of single EATs embedded in a collagen scaffold cultivated without (A) and with mechanical stimulation (B) for 12 days

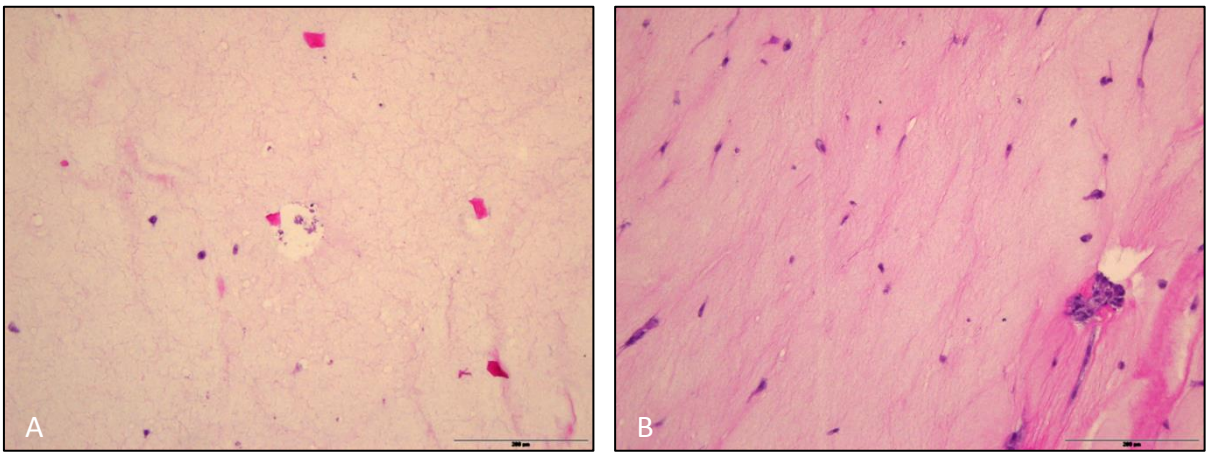


Fig. 32: H&E stained paraffin wax section of MTs composed of EATs embedded in a collagen scaffold cultivated without (A) and with mechanical stimulation (B) for 12 days

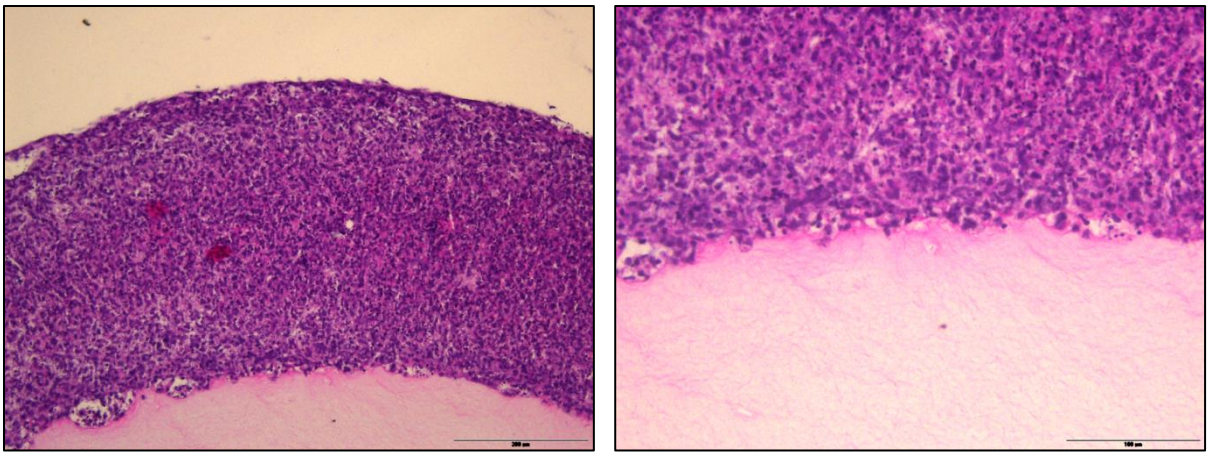


Fig. 33: H&E stained paraffin wax section of straightened ring shaped tissue construct embedded in the collagen scaffold after 12 days of mechanical stimulation

Based on the findings of this pilot study the STREX-ST-140™ appeared to be suitable for further evaluation of the MTs and was therefore purchased for further experiments. Production of the silicon chambers was accompanied by a flat learning curve and was somewhat time consuming, but was finally established. As no incubator with a pull-trough working channel was available at the Center for Applied Biotechnology and Molecular Medicine (CABMM), the new bioreactor was set up at the Center for Clinical Studies at the Tierspital Zürich. Cell cultivation, MT production and loading of the silicon chambers with the collagen scaffold, single cells or MTs was performed at the CABMM. After collagen gel had polymerized, chambers were transported to the Center for Clinical Studies. First experiments with single EATs revealed a cell-concentration dependent shrinkage of the collagen scaffold (Fig. 34a), which was not observed in the pilot study conducted at the ETH. Time of onset of mechanical stimulation had no influence on the amount of shrinkage (Fig. 34b).

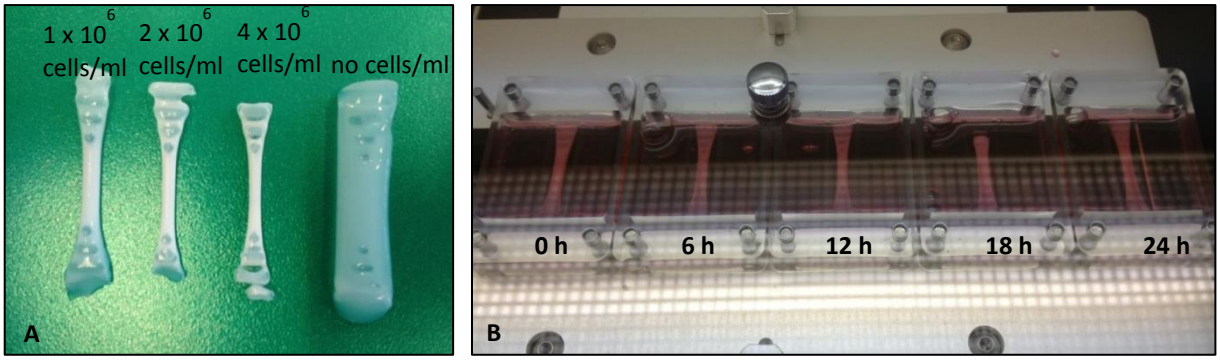


Fig. 34: (A) cell-concentration dependend shrinkage of the collagen scaffold after 4 days; (B) comparable shrinkage of the collagen scaffolds irrespective of onset of mechanical stimulation after 2 days

Even though a lot of effort was undertaken, it was not possible to attain results with the MTs in the new bioreactor, as were obtained in the pilot study at the ETH, Zürich. Irrespective of duration, amount, rest period and strain rates of mechanical stimulation or collagen concentration of the scaffold, cells within the MTs underwent apoptosis, leaving empty holes in the collagen scaffold as seen by histologic evaluation (Fig. 35).

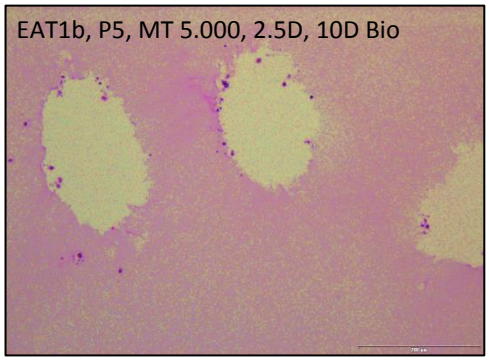


Fig. 35: H&E stained paraffin wax section of the collagen scaffold with empty holes initially filled by MTs after 10 days of mechanical stimulation

To rule out insufficient gas exchange for the cells in the MTs embedded in the collagen scaffold, a new larger lid for the bioreactor was constructed, containing filter protected openings. Temperature within the bioreactor was determined not to exceed 37 °C with the incorporated cooling system. Special attempts were also made to keep the pH of the collagen between 7.2 – 7.4. As experiments with MTs embedded in collagen scaffolds in the silicon chambers under static tension revealed favorable results when carried out at the laboratory of the CABMM, but not when carried out at the Center for Clinical Studies, it was concluded that transportation of the cells in the MTs embedded in the collagen scaffold were most likely to blame for the poor outcome.

6 Overall discussion

Injuries of the weight bearing flexor tendons are one of the most common forms of musculoskeletal injuries that occur in horses (1-3, 5), with a significant impact on athletic performance and animal welfare. This is not only true for race horses, but also for horses performing in other sporting disciplines as well as pleasure horses (6-11). Even though different sporting disciplines place different loads on the locomotor system, it is mainly the SDFT and the SL, which are the main energy storing tendons being most commonly affected (2, 5, 11). Their limited adaptive capacity after skeletal maturation (5, 50), their operation close to their functional limits (43) and the accumulation of age and exercise related degenerative changes (52) are discussed as major predisposing factors. The long convalescence period after injury and the insufficient healing response of the tendon leading to the formation of mechanical inferior scar tissue, and subsequent high re-injury rate, have stimulated ongoing research to improve tendon healing for the last decades. Unfortunately, none of the proposed treatment modalities have so far proven to be superior to conventional treatment (131, 132, 134, 135, 138, 143-145, 147, 148, 230). The use of MSCs or growth factors by means of PRP currently represent two of the most investigated clinical treatment regimes for tendon injury, with marketing being ahead of scientific evidence.

The significance of tendon injury is not only limited to horses but is also of paramount importance in humans (12, 13), where the Achilles and patellar tendon, the rotator cuff and the lateral and medial elbow tendon are the most commonly injured structures (14-16). With an increase in the aged population and participation in recreational sporting activities, the incidence of tendon injuries in humans has risen in the last decades (18, 19), thereby prompting improvements in treatment modalities for tendon healing.

As no single animal model can fully reproduce the naturally occurring situation, several animal models may have to be used in a concerted effort to study the different aspects of tendon injury and healing. Because of ethical concerns about animal welfare and the necessity to follow the principles of the three Rs (replacement, reduction and refinement), many basic research questions may possibly be better answered with *in vitro* experiments. This becomes more likely as new culture formats become available, which more closely resemble the *in vivo* environment, thereby generating more reliable data. *In vitro* culture of tendon-derived fibroblasts allows for the characterization of tendon cells and the monitoring of their response to different treatment modalities. Furthermore, it may ultimately lead to the development of new cellular-based tools to support tendon repair through tissue engineering approaches.

Unfortunately, basic tendon research is hampered by the lack of tendon specific markers. For this reason, the genetic characterization of tendon tissue is normally supplemented with histological analyses, and where feasible, mechanical testing. Tendon-derived fibroblasts in monolayer culture typically exhibit a fibroblast-like appearance, making it impossible to differentiate tendon-derived cells from other fibroblastic cells. Furthermore, in monolayer culture they display an unstable phenotype and tend to dedifferentiate (262, 310-312), which serves to undermine the reliability of the generated data. 3-D cell culture formats, different growth factors and mechanical stimuli have been shown to have an impact on the state of differentiation of tendon-derived fibroblasts *in vitro* (214, 262, 263, 268-270, 337, 341, 342). Therefore, the present study described herein aimed to characterize equine tendon cells under a 3-D culture system, and evaluate the most appropriate stimuli with which to maintain functional differentiated tenocytes *in vitro*.

Gravity assisted assembly of equine adult tenocytes (EATs) and equine fetal tenocytes (EFTs) using the hanging drop system led to the formation of microtissue spheroids (MTs). The size of the MTs was dependent on cell concentration and culture duration, with a decrease in size over time. Whereas apoptosis of cells was a major finding in MTs composed of EFTs, especially at later time points, this was not the case in MTs composed of EATs. Adult tenocytes formed well-organized, viable MTs where cells, especially at late stage cultures, appeared to have an elongated, spindle shaped tenocyte-like morphology and a basic cell alignment growth pattern. Even though, fetal tendon cells were isolated at the end of gestation, the discrepancy in viability of EFTs observed in this culture format points to major differences between adult and fetal tenocytes. As undifferentiated equine MSCs used in this study, as well as mouse ASCs used in other studies (325, 326), had the capacity to form viable MTs, this difference is most likely not just a consequence of insufficient EFTs differentiation. Nutrient supply is a limiting factor for prolonged hanging drop culture, as medium cannot easily be exchanged in the Terasaki plates. This may necessitate early usage of MTs for possible tissue engineering strategies. As EFTs grew well in monolayer culture, but showed increased rates of apoptosis in the MT format, an inappropriate nutrient supply possibly due to higher demands of EFTs should be considered. As fetal cells in general have proven great potential for regenerative therapies (347-351), further evaluation of EFTs is necessary to determine their possible application in tendon regeneration.

MTs composed of EATs demonstrated an early significant increase in tendon-associated marker expression such as *COL1A2*, *COL3A1* and *SCX* when compared to tenocyte monolayers. Unfortunately, they were unable to maintain these elevated levels after prolonged culture times. Also Schulze-Tanzil et al. and Stoll et al. noticed a decrease in type I collagen expression levels in

high-density cultures of human tenocytes over time (262, 263). In the present study expression levels of scleraxis were higher in MTs as compared to monolayer cultures at the different time points. This finding is in accordance with Stoll et al. who also found increased expression levels in 3-D high-density cultures as compared to monolayer cultures (262). In contrast to the study by Schulze-Tanzil et al. (263), expression levels of scleraxis did not increase over time in the MT format.
Interestingly, in the current study, expression levels of the pro-chondrogenic marker *SOX9* were initially observed as being significantly increased in MTs as compared to monolayer cultures after 2 days. However, by day 6 *SOX9* expression levels had become significantly reduced in MTs as compared to monolayers. It is well known, that tenocytes are able to transdifferentiate to fibrochondrocytes under specific culture conditions (352, 353), and that overexpression of *SOX9* converts tenocytes to chondrocytes (354). Furthermore, both *SOX9* and *COL2A1* have been reported as being expressed in native tendon, as well as in tenocytes *in vitro* (263, 265, 266, 355, 356). Importantly, *COL2A1* expression was not detectable at any time points in our culture system, and histologic evaluation of MTs revealed no evidence of cells with chondrocyte-like morphology.
The observed increase in *SCX* and *COL21A2* by the tenocytes in the 3-D MT format, suggest that this culture system has an immediate beneficial effect on tenocyte differentiation as compared to monolayer culture. Unfortunately, this beneficial effect could not be fully maintained over extended periods of culture and no detectable levels of tenomodulin (*TNMD*), another tendon associated marker, were present at any time point tested.
In order to better optimize the tenogenic status of tenocytes cultured under either 2-D or 3-D conditions, we next focused our attention on the growth media used in each system. Findings from recent studies have strongly hinted at the use of TGF-β and IGF-1, or combinations thereof, to enhance and possibly even maintain tenocyte differentiation during extended periods of cultivation. Caliari *et al.* (341) demonstrated that IGF-1 at concentrations ranging from 10 to 200 ng/ml was capable of significantly enhancing *COL1A2*, *COL3A1* and *DCN* gene expression in equine tenocytes cultured in collagen scaffolds in the absence of serum for 7 days. By contrast, a study performed by Qiu *et al.* (270) demonstrated that IGF-1 (50 ng/ml) alone was unable to significantly influence *COL1*, *SCX* or *TNMD* expression in human tenocytes. However, incubation of cells with TGF-β3 (10 ng/ml), or TGF-β3 and IGF-1 together, significantly enhanced gene expression above that of control cultures incubated in the presence of serum-supplemented growth medium. In support of this, studies investigating the tenogenic potential of primary canine MSCs revealed that combinations of TGF-β1 and IGF-1 (5 ng/ml of each) were sufficient to induce the expression of *COL1*, *COL3*, *DCN*, *SCX* and *TNMD* after 7 and 14 days in combination with high density 3-D culture conditions (264). Similarly, a

more recent report by Barsby *et al.* (95) has confirmed a synergistic effect between 3-D growth environments and the addition of TGF-β3 (20 ng/ml) in the tenogenic differentiation of equine ESCs. Therefore, based on these previous findings, we elected to assess the effects of low-serum media containing human TGF-β1 (10 ng/ml) in combination with either human IGF-1 (50 ng/ml) (termed TDM-I), or human insulin and L-ascorbic acid 2-phosphate (termed TDM-II) on tenocyte differentiation in either 2-D or 3-D culture systems. Treatment of tenocytes in 2-D with TDM-I had a moderate effect on most of the genetic markers tested, with the majority of genes being significantly increased within the first 2 days of culture as compared to cells cultured in growth medium alone. However, induction of *TNMD* expression was not observed at any time point, and *COL1A2* and *COL3A1* expression levels were reduced by day 6. With regards to 3-D cultures, early significant increases in gene expression were limited to *SCX* only, but at later stages included increases in *COL1A2*, *COL3A1*, *SCX*, *TNMD* and *SOX9*. In comparison to 2-D cultures, late stage 3-D cultures demonstrated modest benefits in terms of *COL1A2*, *COL3A1* and *TNMD* expression, with similar levels of *SOX9* being observed. These observations therefore go someway to supporting the concept of culturing tenocytes under 3-D conditions in the presence of TGF-β and IGF-1 in order to enhance and maintain a tendon like genotype. However, the most pronounced effects were observed in culture medium where IGF-1 had been replaced by insulin and L-ascorbic acid 2-phosphate (TDM-II). Regardless of which system was used, cultivation in TDM-II resulted in marked increases in the expression of all tenogenic gene markers in late stage cultures.

Furthermore, in comparison to 2-D cultures, 3-D MTs examined at day 6 demonstrated significantly enhanced expression levels of *COL3A1*, *SCX* and *TNMD*, with a noticeable decrease in the expression of the chondrogenic marker *SOX9*, and a complete absence of *COL2A1*. Moreover, not only were the expression levels of tenogenic markers maintained in this medium, but were also in some cases enhanced at the later stages. Clearly therefore, the combination of TGF-β1, insulin and ascorbic acid appears to represent a novel supplement for the purpose of enhancing and maintaining a tendon-like genotype in 3-D tenocyte cultures, without inducing chondrocyte- associated gene expression.

A possible explanation for the increased and more rapid expression of tendon associated markers in the 3-D cultures as compared to monolayer cultures, was that the 3-D conditions allowed for a more advanced state of tenocyte differentiation, thereby altering their response to the growth media. Furthermore, cell surface proteoglycans, which have been shown to act as co-receptors or to modulate the response of cells to pericellular matrix molecules and soluble stimuli, have been shown to be upregulated in 3-D culture formats. Sawaguchi et al. (268) were able to show a significant increase in the expression of syndecan-2, a regulator of TGF-β signaling, in tenocytes cultured under

3-D conditions. In addition, they were also able to show that betaglycan, a TGF-β receptor III, production and betaglycan gene expression were suppressed in monolayer culture.
The TGF-βs induce both *SCX* and *SOX9* expression. The divergent pro-fibrogenic or pro-chondrogenic influence of this signaling pathway is mediated by a stage-dependent regulation of transcriptional signaling repressors that mediate the effects of TGF-β and promote chondrogenesis or fibrogenesis. In the study by Lorda-Diez et al. (357), TGF-interacting factor 1 (Tgif1) was identified as a potential candidate that may be responsible for the pro-fibrogenic influence of TGF-β. Therefore, the transient upregulation of *SOX9* observed in the present study may be explained by alterations in TGF-β signaling, although further studies are needed to confirm this.
With regards to IGF-1, it is expressed in uninjured flexor tendons (107) and is upregulated after tendon injury and repair (99). In the study by Herchenhan et al., IGF-1 supplementation of engineered human tendon tissue imparted a stimulatory effect on fibril diameter and the expression of several genes including *COL1A*, *TNMD* and *SCX in vitro* (109). Similarly, we observed a significant increase in *TNMD* expression following IGF treatment, being observed after 6 days of treatment under 3-D conditions. However, this effect could be significantly increased following the replacement of IGF-1 with insulin, another member of the insulin-like growth factor family, and ascorbic acid. Already in 1977, Schwarz et al. (358) demonstrated that ascorbic acid stabilizes the differentiation of primary avian tenocytes by helping to restore collagen production *in vitro*. A literature search revealed no results concerning an established positive influence of insulin on gene expression or state of differentiation of tenocytes. Interestingly an US patent ((US7985408 B2) covering a tenocyte cell culturing method with the supplementation of insulin was found. Mazzocca et al. investigated the tenogenic potential of insulin in comparison to IGF-1, bFGF or GDF5 on human BMSCs. They found increased gene expression and protein levels of type I and type III collagen, scleraxis, tenascin-C and decorin in BMSCs stimulated with a single dose of insulin in comparison to untreated controls. Expression levels of tendon-associated markers and the corresponding protein levels were comparable or even increased in insulin stimulated cells compared to cells treated with IGF-1, bFGF or GDF5 (359).
We could also demonstrate that type I collagen was also increased at the protein level in tenocyte MTs cultured in the presence of TDM-II. Furthermore, when assessing protein lysates for the presence of TNMD, we detected an additional, high molecular weight protein not observed in MTs treated with growth medium alone. It is unclear as to why TNMD was detected in tenocyte cultures treated with growth medium at all, as qRT-PCR analysis failed to detect any *TNMD* mRNA transcript. However, a similar finding was also reported by Barsby *et al.* (95) in which TNMD protein was

observed in TGF-β-treated equine ESC cultures in the absence of any detectable levels of *TNMD* mRNA. The cause for such inconsistencies was accredited to disparities between TNMD protein turnover and mRNA degradation rates. Therefore, it seems plausible that the 250 kDa band observed in the current study represents a high molecular weight variant of TNMD, being present in native tendon tissue, as well as being induced in MTs following stimulation with TDM-II. However, the relevance of this particular protein species with regards to the normal functioning of tendons remains to be determined. In a final series of experiments, we assessed the potential of tenocytes within MTs to migrate out from the tissue structures and to re-populate 2-D or 3-D culture environments. This was based on the assumption that should such tissue constructs be considered as a viable *in vivo* treatment strategy, then the cells contained within the tissue spheroids would be required to grow out and invade the surrounding host tissue in order to mediate their therapeutic effects. Indeed, we could demonstrate that tenocytes in MTs treated with TDM-II were capable of growing out from the tissues and could proliferate on a 2-D culture surface whilst maintaining a tenocyte-like morphology. Moreover, when transferred to collagen gels, MT-derived tenocytes treated with TDM-II also had the capacity to promote contraction of the collagen matrix and were observed to independently align with respect to the orientation of the collagen structure following MT outgrowth. Furthermore, the contraction rate of the collagen was directly dependent on cell concentration. Other investigators have also observed similar findings using MSCs (360). Therefore, the higher rate of contraction seen in MTs treated with TDM-II is most likely due to increased numbers of viable tenocytes within the collagen scaffold, following their migration out of the MTs.

6.1 Outlook / Perspectives

The generation and culture of equine adult tendon cells in hanging drop cultures represents an interesting 3-D culture method, which may help to maintain tenocytes in a differentiated state, especially when combined with the appropriate growth factor supplementation. In this regard, culture medium containing TGF-β1, insulin and ascorbic acid appears to be superior to medium supplemented with TGF-β1 and IGF-1 only. Clearly, further studies are needed to optimize growth factor supplementation and to elucidate the underlying mechanisms responsible for these effects. Using Terasaki plates for the hanging drop culture makes media change unfeasible, thereby limiting the time of culture period possible. Culture time could be expanded using fully automated systems for hanging drop culture, as provided, for example, by InSphero AG. On the other hand, MTs containing differentiated tenocytes can be easily harvested after complete assembly from Terasaki plates and could be utilized for tissue engineering approaches.

Mechanical stimulation is known to have an impact on gene expression and cell differentiation (69, 218, 219, 342), being partly mediated through TGF-β signaling (214). Therefore, it would have been interesting to characterize equine tenocytes in the MT format, with and without the addition of different growth factors under the influence of mechanical stimulation. Furthermore, mechanical stimulation has been shown to be necessary for embryonic tendon development and therefore may have prevented apoptosis in MTs composed of EFTs. As fetal cells in general have proven great potential for regenerative therapies (347-351), further evaluation of EFTs is necessary to determine their possible application in tendon regeneration.

Unfortunately, it was not possible to modify the first custom made bioreactor in a way to allow for the cultivation of tenocytes under appropriate mechanical stimulation. Although time consuming and quite frustrating, we opted for a different system, the STREX-ST-140™ (from B-Bridge International) which seemed a feasible option following a successful pilot study performed in cooperation with the Institute for Biomechanics (ETH, Zurich). However, despite our best efforts, we were unable to reproduce these results and as such, had to compromise and opt for a static 3-D culture. Therefore, further optimization studies are required in order to allow for the STREX-ST-140™ to be used to evaluate the effects of mechanical stimulation on the ability of MT tenocytes to repopulate collagen scaffolds and to generate tendon tissue.

In addition to equine tenocytes, we also observed a positive influence of the hanging drop culture system on the differentiation capacity of equine MSCs. The osteogenic and adipogenic differentiation of equine MSCs appeared to proceed faster in this 3-D culture format as compared to monolayer culture. Therefore the MT format with the addition of different growth factors could be adapted to investigate possible tenogenic differentiation of MSCs. Furthermore, with an established bioreactor system, the influence of mechanical stimulation on stem cell differentiation could also be elucidated. Co-cultures with different ratios of EATs and EFTs were also feasible. Although one proposed mode of action by which MSCs could enhance tendon healing is through the release of different stimulatory factors, we were unable to observe any positive influence of tenocyte/MSC co-cultures on the expression levels of tendon associated markers. Further and more refined experiments are needed to explore this question.

In conclusion our findings demonstrate the beneficial effect of culturing EATs as 3-D gravity enforced MTs and supports the use of low-serum containing growth medium in combination with TGF-β1, insulin and ascorbic acid as an effective means by which to maintain the tenogenic differentiation status. Such a system would not only offer potential benefits for tendon tissue formation *in vivo*, but also just as importantly allow for tendon cell and tissue biology to be studied *in vitro* using conditions

that more closely simulate the *in vivo* situation. This would undoubtedly have a significant impact on *in vivo* experimentation, reducing the number of animals required and thus upholding the philosophy of the 3Rs. Furthermore, the hanging drop culture offers a valid system to explore the interaction between tenocytes and MSCs and may therefore help to elucidate a possible mode of action by which MSCs act to promote tendon healing. Finally, the influence of mechanical cues on the tenogenic differentiation of tenocyte MTs needs to be further explored.

7 References

1. Williams RB, Harkins LS, Hammond CJ, Wood JL. Racehorse injuries, clinical problems and fatalities recorded on British racecourses from flat racing and National Hunt racing during 1996, 1997 and 1998. Equine veterinary journal. 2001;33(5):478-86. Epub 2001/09/18.
2. Ely ER, Verheyen KL, Wood JL. Fractures and tendon injuries in National Hunt horses in training in the UK: a pilot study. Equine veterinary journal. 2004;36(4):365-7. Epub 2004/05/28.
3. Pinchbeck GL, Clegg PD, Proudman CJ, Stirk A, Morgan KL, French NP. Horse injuries and racing practices in National Hunt racehorses in the UK: the results of a prospective cohort study. Veterinary journal. 2004;167(1):45-52. Epub 2003/11/19.
4. Lam KH, Parkin TD, Riggs CM, Morgan KL. Descriptive analysis of retirement of Thoroughbred racehorses due to tendon injuries at the Hong Kong Jockey Club (1992-2004). Equine veterinary journal. 2007;39(2):143-8. Epub 2007/03/24.
5. Kasashima Y, Takahashi T, Smith RK, Goodship AE, Kuwano A, Ueno T, et al. Prevalence of superficial digital flexor tendonitis and suspensory desmitis in Japanese Thoroughbred flat racehorses in 1999. Equine veterinary journal. 2004;36(4):346-50. Epub 2004/05/28.
6. Sloet van Oldruitenborgh-Oosterbaan MM, Genzel W, van Weeren PR. A pilot study on factors influencing the career of Dutch sport horses. Equine veterinary journal Supplement. 2010(38):28-32. Epub 2011/05/27.
7. Murray RC, Dyson SJ, Tranquille C, Adams V. Association of type of sport and performance level with anatomical site of orthopaedic injury diagnosis. Equine veterinary journal Supplement. 2006(36):411-6. Epub 2007/04/04.
8. van den Belt AJ, Dik KJ, Barneveld A. Ultrasonographic evaluation and long term follow-up of flexor tendonitis/desmitis in the metacarpal/metatarsal region in Dutch warmblood horses and standardbred racehorses. The Veterinary quarterly. 1994;16(sup2):76-80. Epub 1994/05/01.
9. Gibson KT, Snyder JR, Spier SJ. Ultrasonographic diagnosis of soft tissue injuries in horses competing at the Sydney 2000 Olympic Games. Equine Vet Educ. 2002;14(3):149-56.
10. Murray RC, Walters JM, Snart H, Dyson SJ, Parkin TD. Identification of risk factors for lameness in dressage horses. Veterinary journal. 2010;184(1):27-36. Epub 2009/04/17.
11. Trump M. A retrospective study of the prevalence of injuries to the suspensory ligament, digital flexor tendons and associated structures in a non-racehorse referral-hospital population. Switzerland: University of Zurich; 2014.
12. McCormick A, Charlton J, Fleming D. Assessing health needs in primary care. Morbidity study from general practice provides another source of information. Bmj. 1995;310(6993):1534. Epub 1995/06/10.
13. Jarvinen TA, Kannus P, Maffulli N, Khan KM. Achilles tendon disorders: etiology and epidemiology. Foot and ankle clinics. 2005;10(2):255-66. Epub 2005/06/01.
14. Kujala UM, Sarna S, Kaprio J. Cumulative incidence of achilles tendon rupture and tendinopathy in male former elite athletes. Clinical journal of sport medicine : official journal of the Canadian Academy of Sport Medicine. 2005;15(3):133-5. Epub 2005/05/04.
15. Kannus P, Aho H, Jarvinen M, Niittymaki S. Computerized recording of visits to an outpatient sports clinic. The American journal of sports medicine. 1987;15(1):79-85. Epub 1987/01/01.
16. Woodley BL, Newsham-West RJ, Baxter GD. Chronic tendinopathy: effectiveness of eccentric exercise. British journal of sports medicine. 2007;41(4):188-98; discussion 99. Epub 2006/10/26.
17. Gruchow HW, Pelletier D. An epidemiologic study of tennis elbow. Incidence, recurrence, and effectiveness of prevention strategies. The American journal of sports medicine. 1979;7(4):234-8. Epub 1979/07/01.
18. Rolf C, Movin T. Etiology, histopathology, and outcome of surgery in achillodynia. Foot & ankle international. 1997;18(9):565-9. Epub 1997/10/06.

19. Kader D, Saxena A, Movin T, Maffulli N. Achilles tendinopathy: some aspects of basic science and clinical management. British journal of sports medicine. 2002;36(4):239-49. Epub 2002/07/30.
20. van Dijk CN, van Sterkenburg MN, Wiegerinck JI, Karlsson J, Maffulli N. Terminology for Achilles tendon related disorders. Knee surgery, sports traumatology, arthroscopy : official journal of the ESSKA. 2011;19(5):835-41. Epub 2011/01/12.
21. Mienaltowski MJ, Birk DE. Structure, physiology, and biochemistry of collagens. Adv Exp Med Biol. 2014;802:5-29.
22. Smith RKW. Pathophysiology of Tendon Injury. In: Ross MW, Dyson SJ, editors. Diagnosis and management of lameness in the horse. Philadelphia, Pa. ; London: W. B. Saunders; 2003. p. 616-28.
23. Wilson AM, McGuigan MP, Su A, van Den Bogert AJ. Horses damp the spring in their step. Nature. 2001;414(6866):895-9. Epub 2002/01/10.
24. Alexander RM. Energy-saving mechanisms in walking and running. The Journal of experimental biology. 1991;160:55-69. Epub 1991/10/01.
25. Wang JH, Iosifidis MI, Fu FH. Biomechanical basis for tendinopathy. Clinical orthopaedics and related research. 2006;443:320-32. Epub 2006/02/08.
26. Goodship AE, Birch HL, Wilson AM. The pathobiology and repair of tendon and ligament injury. The Veterinary clinics of North America Equine practice. 1994;10(2):323-49. Epub 1994/08/01.
27. Smith RKW, Webbon PM, editors. The Physiology of Normal Tendon and Ligament. Dubai Equine International Symposium: The Equine Athlete-Tendon, Ligament and Soft Tissue Injuries; 1996 1996; Dubai, United Arab Emirates.
28. Stanley RL, Fleck RA, Becker DL, Goodship AE, Ralphs JR, Patterson-Kane JC. Gap junction protein expression and cellularity: comparison of immature and adult equine digital tendons. Journal of anatomy. 2007;211(3):325-34. Epub 2007/09/13.
29. Einhorn TA, O'Keefe, R.J., Buckwalter, J.A., editor. Orthopaedic basic science. 3rd ed. Rosemont: American Academy of Orthopaedic Surgeons; 2007.
30. Avella CS, Smith RKW. Diagnosis and Management of Tendon and Ligament Disorders. In: Stick JA, Auer JA, editors. Equine Surgery 4th ed. Saint Louis: W.B. Saunders; 2012. p. 1157-79.
31. Schatzker J, Branemark PI. Intravital observations on the microvascular anatomy and microcirculation of the tendon. Acta orthopaedica Scandinavica Supplementum. 1969;126:1-23. Epub 1969/01/01.
32. Hoffmann A, Gross G. Tendon and ligament engineering in the adult organism: mesenchymal stem cells and gene-therapeutic approaches. International orthopaedics. 2007;31(6):791-7. Epub 2007/07/20.
33. Ansorge HL, Meng X, Zhang G, Veit G, Sun M, Klement JF, et al. Type XIV Collagen Regulates Fibrillogenesis: PREMATURE COLLAGEN FIBRIL GROWTH AND TISSUE DYSFUNCTION IN NULL MICE. The Journal of biological chemistry. 2009;284(13):8427-38. Epub 2009/01/13.
34. Zhang G, Young BB, Birk DE. Differential expression of type XII collagen in developing chicken metatarsal tendons. Journal of anatomy. 2003;202(5):411-20. Epub 2003/05/13.
35. Smith RK, Zunino L, Webbon PM, Heinegard D. The distribution of cartilage oligomeric matrix protein (COMP) in tendon and its variation with tendon site, age and load. Matrix biology : journal of the International Society for Matrix Biology. 1997;16(5):255-71. Epub 1998/03/21.
36. Smith RK, Gerard M, Dowling B, Dart AJ, Birch HL, Goodship AE. Correlation of cartilage oligomeric matrix protein (COMP) levels in equine tendon with mechanical properties: a proposed role for COMP in determining function-specific mechanical characteristics of locomotor tendons. Equine veterinary journal Supplement. 2002(34):241-4. Epub 2002/10/31.
37. Svensson L, Aszodi A, Heinegard D, Hunziker EB, Reinholt FP, Fassler R, et al. Cartilage oligomeric matrix protein-deficient mice have normal skeletal development. Molecular and cellular biology. 2002;22(12):4366-71. Epub 2002/05/25.
38. Qi J, Fox AM, Alexopoulos LG, Chi L, Bynum D, Guilak F, et al. IL-1beta decreases the elastic modulus of human tenocytes. Journal of applied physiology. 2006;101(1):189-95. Epub 2006/04/22.

39. Stephens PR, Nunamaker DM, Butterweck DM. Application of a Hall-effect transducer for measurement of tendon strains in horses. American journal of veterinary research. 1989;50(7):1089-95. Epub 1989/07/01.
40. Riemersma DJ, Schamhardt HC. In vitro mechanical properties of equine tendons in relation to cross-sectional area and collagen content. Research in veterinary science. 1985;39(3):263-70. Epub 1985/11/01.
41. Dowling BA, Dart AJ, Hodgson DR, Rose RJ, Walsh WR. Recombinant equine growth hormone does not affect the in vitro biomechanical properties of equine superficial digital flexor tendon. Veterinary surgery : VS. 2002;31(4):325-30. Epub 2002/07/03.
42. Gerard MP, Hodgson DR, Rose RJ, Walsh WR. Effects of recombinant equine growth hormone on in vitro biomechanical properties of the superficial digital flexor tendon of Standardbred yearlings in training. Veterinary surgery : VS. 2005;34(3):253-9. Epub 2005/08/24.
43. Dowling BA, Dart AJ, Hodgson DR, Smith RK. Superficial digital flexor tendonitis in the horse. Equine veterinary journal. 2000;32(5):369-78. Epub 2000/10/19.
44. Platt D, Wilson AM, Timbs A, Wright IM, Goodship AE. Novel force transducer for the measurement of tendon force in vivo. J Biomech. 1994;27(12):1489-93. Epub 1994/12/01.
45. Denoix JM. Functional anatomy of tendons and ligaments in the distal limbs (manus and pes). The Veterinary clinics of North America Equine practice. 1994;10(2):273-322. Epub 1994/08/01.
46. Butcher MT, Hermanson JW, Ducharme NG, Mitchell LM, Soderholm LV, Bertram JE. Contractile behavior of the forelimb digital flexors during steady-state locomotion in horses (Equus caballus): an initial test of muscle architectural hypotheses about in vivo function. Comparative biochemistry and physiology Part A, Molecular & integrative physiology. 2009;152(1):100-14. Epub 2008/10/07.
47. Butcher MT, Hermanson JW, Ducharme NG, Mitchell LM, Soderholm LV, Bertram JE. Superficial digital flexor tendon lesions in racehorses as a sequela to muscle fatigue: a preliminary study. Equine veterinary journal. 2007;39(6):540-5. Epub 2007/12/11.
48. Peloso JG, Mundy GD, Cohen ND. Prevalence of, and factors associated with, musculoskeletal racing injuries of thoroughbreds. Journal of the American Veterinary Medical Association. 1994;204(4):620-6. Epub 1994/02/15.
49. Takahashi T, Kasashima Y, Ueno Y. Association between race history and risk of superficial digital flexor tendon injury in Thoroughbred racehorses. Journal of the American Veterinary Medical Association. 2004;225(1):90-3. Epub 2004/07/09.
50. Smith RK, Birch HL, Goodman S, Heinegard D, Goodship AE. The influence of ageing and exercise on tendon growth and degeneration--hypotheses for the initiation and prevention of strain-induced tendinopathies. Comparative biochemistry and physiology Part A, Molecular & integrative physiology. 2002;133(4):1039-50. Epub 2002/12/18.
51. Patterson-Kane JC, Parry DA, Birch HL, Goodship AE, Firth EC. An age-related study of morphology and cross-link composition of collagen fibrils in the digital flexor tendons of young thoroughbred horses. Connective tissue research. 1997;36(3):253-60. Epub 1997/01/01.
52. Pickersgill CH. Epidemiological studies into orthopaedic conditions of the equine athlete. United Kingdom: University of Glasgow; 2000.
53. Haglund-Akerlind Y, Eriksson E. Range of motion, muscle torque and training habits in runners with and without Achilles tendon problems. Knee surgery, sports traumatology, arthroscopy : official journal of the ESSKA. 1993;1(3-4):195-9. Epub 1993/01/01.
54. Birch HL, Bailey AJ, Goodship AE. Macroscopic 'degeneration' of equine superficial digital flexor tendon is accompanied by a change in extracellular matrix composition. Equine veterinary journal. 1998;30(6):534-9. Epub 1998/12/09.
55. Patterson-Kane JC, Wilson AM, Firth EC, Parry DA, Goodship AE. Comparison of collagen fibril populations in the superficial digital flexor tendons of exercised and nonexercised thoroughbreds. Equine veterinary journal. 1997;29(2):121-5. Epub 1997/03/01.

56. Patterson-Kane JC, Firth EC, Goodship AE, Parry DA. Age-related differences in collagen crimp patterns in the superficial digital flexor tendon core region of untrained horses. Australian veterinary journal. 1997;75(1):39-44. Epub 1997/01/01.
57. Patterson-Kane JC, Wilson AM, Firth EC, Parry DA, Goodship AE. Exercise-related alterations in crimp morphology in the central regions of superficial digital flexor tendons from young thoroughbreds: a controlled study. Equine veterinary journal. 1998;30(1):61-4. Epub 1998/02/12.
58. Thorpe CT, Clegg PD, Birch HL. A review of tendon injury: why is the equine superficial digital flexor tendon most at risk? Equine veterinary journal. 2010;42(2):174-80. Epub 2010/02/17.
59. Wilson AM, Goodship AE. Exercise-induced hyperthermia as a possible mechanism for tendon degeneration. J Biomech. 1994;27(7):899-905. Epub 1994/07/01.
60. Birch HL, Wilson AM, Goodship AE. The effect of exercise-induced localised hyperthermia on tendon cell survival. The Journal of experimental biology. 1997;200(Pt 11):1703-8. Epub 1997/06/01.
61. Hosaka Y, Ozoe S, Kirisawa R, Ueda H, Takehana K, Yamaguchi M. Effect of heat on synthesis of gelatinases and pro-inflammatory cytokines in equine tendinocytes. Biomedical research. 2006;27(5):233-41. Epub 2006/11/14.
62. Tsuzaki M, Bynum D, Almekinders L, Yang X, Faber J, Banes AJ. ATP modulates load-inducible IL-1beta, COX 2, and MMP-3 gene expression in human tendon cells. Journal of cellular biochemistry. 2003;89(3):556-62. Epub 2003/05/23.
63. Tsuzaki M, Guyton G, Garrett W, Archambault JM, Herzog W, Almekinders L, et al. IL-1 beta induces COX2, MMP-1, -3 and -13, ADAMTS-4, IL-1 beta and IL-6 in human tendon cells. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2003;21(2):256-64. Epub 2003/02/06.
64. Wang JH, Jia F, Yang G, Yang S, Campbell BH, Stone D, et al. Cyclic mechanical stretching of human tendon fibroblasts increases the production of prostaglandin E2 and levels of cyclooxygenase expression: a novel in vitro model study. Connective tissue research. 2003;44(3-4):128-33. Epub 2003/09/25.
65. Clegg PD, Strassburg S, Smith RK. Cell phenotypic variation in normal and damaged tendons. International journal of experimental pathology. 2007;88(4):227-35. Epub 2007/08/19.
66. Lo IK, Marchuk LL, Hollinshead R, Hart DA, Frank CB. Matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase mRNA levels are specifically altered in torn rotator cuff tendons. The American journal of sports medicine. 2004;32(5):1223-9. Epub 2004/07/21.
67. Jones GC, Corps AN, Pennington CJ, Clark IM, Edwards DR, Bradley MM, et al. Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human achilles tendon. Arthritis and rheumatism. 2006;54(3):832-42. Epub 2006/03/02.
68. Arnoczky SP, Lavagnino M, Egerbacher M, Caballero O, Gardner K, Shender MA. Loss of homeostatic strain alters mechanostat "set point" of tendon cells in vitro. Clinical orthopaedics and related research. 2008;466(7):1583-91. Epub 2008/05/07.
69. Egerbacher M, Arnoczky SP, Caballero O, Lavagnino M, Gardner KL. Loss of homeostatic tension induces apoptosis in tendon cells: an in vitro study. Clinical orthopaedics and related research. 2008;466(7):1562-8. Epub 2008/05/07.
70. Williams IF, Heaton A, McCullagh KG. Cell morphology and collagen types in equine tendon scar. Research in veterinary science. 1980;28(3):302-10. Epub 1980/05/01.
71. Maffulli N, Ewen SW, Waterston SW, Reaper J, Barrass V. Tenocytes from ruptured and tendinopathic achilles tendons produce greater quantities of type III collagen than tenocytes from normal achilles tendons. An in vitro model of human tendon healing. The American journal of sports medicine. 2000;28(4):499-505. Epub 2000/08/02.
72. Eriksen HA, Pajala A, Leppilahti J, Risteli J. Increased content of type III collagen at the rupture site of human Achilles tendon. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2002;20(6):1352-7. Epub 2002/12/11.

73. Maffulli N, Barrass V, Ewen SW. Light microscopic histology of achilles tendon ruptures. A comparison with unruptured tendons. The American journal of sports medicine. 2000;28(6):857-63. Epub 2000/12/02.
74. Rufai A, Ralphs JR, Benjamin M. Structure and histopathology of the insertional region of the human Achilles tendon. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 1995;13(4):585-93. Epub 1995/07/01.
75. Gelberman RH, Vandeberg JS, Manske PR, Akeson WH. The early stages of flexor tendon healing: a morphologic study of the first fourteen days. The Journal of hand surgery. 1985;10(6 Pt 1):776-84. Epub 1985/11/01.
76. Manske PR, Gelberman RH, Lesker PA. Flexor tendon healing. Hand Clin. 1985;1(1):25-34. Epub 1985/02/01.
77. Manske PR, Lesker PA, Gelberman RH, Rucinsky TE. Intrinsic restoration of the flexor tendon surface in the nonhuman primate. The Journal of hand surgery. 1985;10(5):632-7. Epub 1985/09/01.
78. Wang JH. Mechanobiology of tendon. J Biomech. 2006;39(9):1563-82. Epub 2005/07/08.
79. Pufe T, Petersen W, Kurz B, Tsokos M, Tillmann B, Mentlein R. Mechanical factors influence the expression of endostatin--an inhibitor of angiogenesis--in tendons. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2003;21(4):610-6. Epub 2003/06/12.
80. Pufe T, Petersen WJ, Mentlein R, Tillmann BN. The role of vasculature and angiogenesis for the pathogenesis of degenerative tendons disease. Scandinavian journal of medicine & science in sports. 2005;15(4):211-22. Epub 2005/07/07.
81. Matthews P, Richards H. The repair potential of digital flexor tendons. An experimental study. The Journal of bone and joint surgery British volume. 1974;56-B(4):618-25. Epub 1974/11/01.
82. Potenza AD. Tendon healing within the flexor digital sheath in the dog. The Journal of bone and joint surgery American volume. 1962;44-A:49-64. Epub 1962/01/01.
83. Lundborg G. Experimental flexor tendon healing without adhesion formation--a new concept of tendon nutrition and intrinsic healing mechanisms. A preliminary report. Hand. 1976;8(3):235-8.
84. Gelberman RH, Manske PR, Akeson WH, Woo SL, Lundborg G, Amiel D. Flexor tendon repair. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 1986;4(1):119-28. Epub 1986/01/01.
85. Wong JK, Lui YH, Kapacee Z, Kadler KE, Ferguson MW, McGrouther DA. The cellular biology of flexor tendon adhesion formation: an old problem in a new paradigm. The American journal of pathology. 2009;175(5):1938-51. Epub 2009/10/17.
86. Lin TW, Cardenas L, Soslowsky LJ. Biomechanics of tendon injury and repair. J Biomech. 2004;37(6):865-77. Epub 2004/04/28.
87. James R, Kesturu G, Balian G, Chhabra AB. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. The Journal of hand surgery. 2008;33(1):102-12. Epub 2008/02/12.
88. Kajikawa Y, Morihara T, Watanabe N, Sakamoto H, Matsuda K, Kobayashi M, et al. GFP chimeric models exhibited a biphasic pattern of mesenchymal cell invasion in tendon healing. Journal of cellular physiology. 2007;210(3):684-91. Epub 2006/12/13.
89. Yang G, Rothrauff BB, Tuan RS. Tendon and ligament regeneration and repair: clinical relevance and developmental paradigm. Birth defects research Part C, Embryo today : reviews. 2013;99(3):203-22. Epub 2013/10/01.
90. Evans CH. Cytokines and the role they play in the healing of ligaments and tendons. Sports medicine. 1999;28(2):71-6. Epub 1999/09/24.
91. Beredjiklian PK. Biologic aspects of flexor tendon laceration and repair. The Journal of bone and joint surgery American volume. 2003;85-A(3):539-50. Epub 2003/03/15.

92. Chen CH, Zhou YL, Wu YF, Cao Y, Gao JS, Tang JB. Effectiveness of microRNA in Down-regulation of TGF-beta gene expression in digital flexor tendons of chickens: in vitro and in vivo study. The Journal of hand surgery. 2009;34(10):1777-84 e1. Epub 2009/12/09.
93. Tsubone T, Moran SL, Amadio PC, Zhao C, An KN. Expression of growth factors in canine flexor tendon after laceration in vivo. Annals of plastic surgery. 2004;53(4):393-7. Epub 2004/09/24.
94. Ngo M, Pham H, Longaker MT, Chang J. Differential expression of transforming growth factor-beta receptors in a rabbit zone II flexor tendon wound healing model. Plastic and reconstructive surgery. 2001;108(5):1260-7. Epub 2001/10/18.
95. Barsby T, Guest D. Transforming growth factor beta3 promotes tendon differentiation of equine embryo-derived stem cells. Tissue engineering Part A. 2013;19(19-20):2156-65. Epub 2013/04/25.
96. Chang J, Most D, Stelnicki E, Siebert JW, Longaker MT, Hui K, et al. Gene expression of transforming growth factor beta-1 in rabbit zone II flexor tendon wound healing: evidence for dual mechanisms of repair. Plastic and reconstructive surgery. 1997;100(4):937-44. Epub 1997/09/18.
97. Khan R, Sheppard R. Fibrosis in heart disease: understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease and arrhythmia. Immunology. 2006;118(1):10-24. Epub 2006/04/25.
98. Bonniaud P, Martin G, Margetts PJ, Ask K, Robertson J, Gauldie J, et al. Connective tissue growth factor is crucial to inducing a profibrotic environment in "fibrosis-resistant" BALB/c mouse lungs. American journal of respiratory cell and molecular biology. 2004;31(5):510-6. Epub 2004/07/17.
99. Chen CH, Cao Y, Wu YF, Bais AJ, Gao JS, Tang JB. Tendon healing in vivo: gene expression and production of multiple growth factors in early tendon healing period. The Journal of hand surgery. 2008;33(10):1834-42. Epub 2008/12/17.
100. Cui Q, Wang Z, Jiang D, Qu L, Guo J, Li Z. HGF inhibits TGF-beta1-induced myofibroblast differentiation and ECM deposition via MMP-2 in Achilles tendon in rat. European journal of applied physiology. 2011;111(7):1457-63. Epub 2010/12/18.
101. Duffy FJ, Jr., Seiler JG, Gelberman RH, Hergrueter CA. Growth factors and canine flexor tendon healing: initial studies in uninjured and repair models. The Journal of hand surgery. 1995;20(4):645-9. Epub 1995/07/01.
102. Berglund M, Reno C, Hart DA, Wiig M. Patterns of mRNA expression for matrix molecules and growth factors in flexor tendon injury: differences in the regulation between tendon and tendon sheath. The Journal of hand surgery. 2006;31(8):1279-87. Epub 2006/10/10.
103. Chang J, Most D, Thunder R, Mehrara B, Longaker MT, Lineaweaver WC. Molecular studies in flexor tendon wound healing: the role of basic fibroblast growth factor gene expression. The Journal of hand surgery. 1998;23(6):1052-8. Epub 1998/12/16.
104. Tang JB, Xu Y, Ding F, Wang XT. Tendon healing in vitro: promotion of collagen gene expression by bFGF with NF-kappaB gene activation. The Journal of hand surgery. 2003;28(2):215-20. Epub 2003/04/03.
105. Tang JB, Cao Y, Zhu B, Xin KQ, Wang XT, Liu PY. Adeno-associated virus-2-mediated bFGF gene transfer to digital flexor tendons significantly increases healing strength. an in vivo study. The Journal of bone and joint surgery American volume. 2008;90(5):1078-89. Epub 2008/05/03.
106. Thomopoulos S, Kim HM, Das R, Silva MJ, Sakiyama-Elbert S, Amiel D, et al. The effects of exogenous basic fibroblast growth factor on intrasynovial flexor tendon healing in a canine model. The Journal of bone and joint surgery American volume. 2010;92(13):2285-93. Epub 2010/10/12.
107. Tsuzaki M, Brigman BE, Yamamoto J, Lawrence WT, Simmons JG, Mohapatra NK, et al. Insulin-like growth factor-I is expressed by avian flexor tendon cells. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2000;18(4):546-56. Epub 2000/10/29.

108. Abrahamsson SO. Similar effects of recombinant human insulin-like growth factor-I and II on cellular activities in flexor tendons of young rabbits: experimental studies in vitro. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 1997;15(2):256-62. Epub 1997/03/01.
109. Herchenhan A, Bayer ML, Eliasson P, Magnusson SP, Kjaer M. Insulin-like growth factor I enhances collagen synthesis in engineered human tendon tissue. Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society. 2015;25(1):13-9. Epub 2014/10/05.
110. Dahlgren LA, van der Meulen MC, Bertram JE, Starrak GS, Nixon AJ. Insulin-like growth factor-I improves cellular and molecular aspects of healing in a collagenase-induced model of flexor tendinitis. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2002;20(5):910-9. Epub 2002/10/18.
111. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, et al. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science. 1989;246(4935):1309-12. Epub 1989/12/08.
112. Zhang F, Liu H, Stile F, Lei MP, Pang Y, Oswald TM, et al. Effect of vascular endothelial growth factor on rat Achilles tendon healing. Plastic and reconstructive surgery. 2003;112(6):1613-9. Epub 2003/10/28.
113. Pufe T, Petersen W, Tillmann B, Mentlein R. The angiogenic peptide vascular endothelial growth factor is expressed in foetal and ruptured tendons. Virchows Archiv : an international journal of pathology. 2001;439(4):579-85. Epub 2001/11/17.
114. Chhabra A, Tsou D, Clark RT, Gaschen V, Hunziker EB, Mikic B. GDF-5 deficiency in mice delays Achilles tendon healing. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2003;21(5):826-35. Epub 2003/08/16.
115. Clark RT, Johnson TL, Schalet BJ, Davis L, Gaschen V, Hunziker EB, et al. GDF-5 deficiency in mice leads to disruption of tail tendon form and function. Connective tissue research. 2001;42(3):175-86. Epub 2002/03/27.
116. Mikic B, Rossmeier K, Bierwert L. Sexual dimorphism in the effect of GDF-6 deficiency on murine tendon. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2009;27(12):1603-11. Epub 2009/06/06.
117. Mendias CL, Bakhurin KI, Faulkner JA. Tendons of myostatin-deficient mice are small, brittle, and hypocellular. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(1):388-93. Epub 2007/12/29.
118. Fu SC, Wong YP, Chan BP, Pau HM, Cheuk YC, Lee KM, et al. The roles of bone morphogenetic protein (BMP) 12 in stimulating the proliferation and matrix production of human patellar tendon fibroblasts. Life sciences. 2003;72(26):2965-74. Epub 2003/04/23.
119. Lou J, Tu Y, Burns M, Silva MJ, Manske P. BMP-12 gene transfer augmentation of lacerated tendon repair. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2001;19(6):1199-202. Epub 2002/01/10.
120. Heisterbach PE, Todorov A, Fluckiger R, Evans CH, Majewski M. Effect of BMP-12, TGF-beta1 and autologous conditioned serum on growth factor expression in Achilles tendon healing. Knee surgery, sports traumatology, arthroscopy : official journal of the ESSKA. 2012;20(10):1907-14. Epub 2011/11/30.
121. Lee JY, Zhou Z, Taub PJ, Ramcharan M, Li Y, Akinbiyi T, et al. BMP-12 treatment of adult mesenchymal stem cells in vitro augments tendon-like tissue formation and defect repair in vivo. PloS one. 2011;6(3):e17531. Epub 2011/03/18.
122. Tan SL, Ahmad RE, Ahmad TS, Merican AM, Abbas AA, Ng WM, et al. Effect of growth differentiation factor 5 on the proliferation and tenogenic differentiation potential of human mesenchymal stem cells in vitro. Cells, tissues, organs. 2012;196(4):325-38. Epub 2012/06/02.

123. Haupt JL, Donnelly BP, Nixon AJ. Effects of platelet-derived growth factor-BB on the metabolic function and morphologic features of equine tendon in explant culture. American journal of veterinary research. 2006;67(9):1595-600.
124. Wang XT, Liu PY, Tang JB. Tendon healing in vitro: modification of tenocytes with exogenous vascular endothelial growth factor gene increases expression of transforming growth factor beta but minimally affects expression of collagen genes. The Journal of hand surgery. 2005;30(2):222-9. Epub 2005/03/23.
125. Bagge J, Lorentzon R, Alfredson H, Forsgren S. Unexpected presence of the neurotrophins NGF and BDNF and the neurotrophin receptor p75 in the tendon cells of the human Achilles tendon. Histology and histopathology. 2009;24(7):839-48. Epub 2009/05/29.
126. Berglund ME, Hart DA, Reno C, Wiig M. Growth factor and protease expression during different phases of healing after rabbit deep flexor tendon repair. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2011;29(6):886-92. Epub 2011/01/20.
127. Jorgensen JS, Genovese RL. Superficil Digital Flexor Tendonitis. In: Ross MW, Dyson SJ, editors. Diagnosis and management of lameness in the horse. Philadelphia, Pa. ; London: W. B. Saunders; 2003. p. 628-43.
128. Asheim A. Surgical Treatment of Tendon Injuries in the Horse. Journal of the American Veterinary Medical Association. 1964;145:447-51.
129. Marr CM, Love S, Boyd JS, McKellar Q. Factors affecting the clinical outcome of injuries to the superficial digital flexor tendon in National Hunt and point-to-point racehorses. The Veterinary record. 1993;132(19):476-9. Epub 1993/05/08.
130. Dyson SJ. Medical management of superficial digital flexor tendonitis: a comparative study in 219 horses (1992-2000). Equine veterinary journal. 2004;36(5):415-9. Epub 2004/07/16.
131. Genovese RL, editor. Prognosis of superficial digital flexor tendon and suspensory ligament injuries. 39th Annual Convention Aerican Association of Equine Practitioners; 1993; San Antonio, Texas.
132. Dahlgren LA, Rosenbusch RF, Booth LC. The effect of polysulphated glycosaminoglycan on the production of proteoglycan by equine tendon fibroblasts in monolayer culture. Vet Comp Orthopaed. 1998;11(3):136-40.
133. Gaughan EM, Nixon AJ, Krook LP, Yeager AE, Mann KA, Mohammed H, et al. Effects of sodium hyaluronate on tendon healing and adhesion formation in horses. American journal of veterinary research. 1991;52(5):764-73. Epub 1991/05/01.
134. Wiig M, Abrahamsson SO, Lundborg G. Effects of hyaluronan on cell proliferation and collagen synthesis: a study of rabbit flexor tendons in vitro. The Journal of hand surgery. 1996;21(4):599-604. Epub 1996/07/01.
135. Dahlgren LA, Nixon AJ, Brower-Toland BD. Effects of beta-aminopropionitrile on equine tendon metabolism in vitro and on effects of insulin-like growth factor-I on matrix production by equine tenocytes. American journal of veterinary research. 2001;62(10):1557-62. Epub 2001/10/11.
136. Foland JW, Trotter GW, Powers BE, Wrigley RH, Smith FW. Effect of sodium hyaluronate in collagenase-induced superficial digital flexor tendinitis in horses. American journal of veterinary research. 1992;53(12):2371-6. Epub 1992/12/01.
137. Genovese RL, Rantanen NW, Hauser ML, Simpson BS. Diagnostic Ultrasonography of Equine Limbs. Vet Clin N Am-Equine. 1986;2(1):145-226.
138. Yamamoto E, Hata D, Kobayashi A, Ueda H, Tangkawattana P, Oikawa M, et al. Effect of beta-aminopropionitrile and hyaluronic acid on repair of collagenase-induced injury of the rabbit Achilles tendon. J Comp Pathol. 2002;126(2-3):161-70.
139. Crowe OM, Dyson SJ, Wright IM, Schramme MC, Smith RK. Treatment of chronic or recurrent proximal suspensory desmitis using radial pressure wave therapy in the horse. Equine veterinary journal. 2004;36(4):313-6.

140. Dyson S. Proximal suspensory desmitis in the hindlimb: 42 cases. British Veterinary Journal. 1994;150(3):279-91.
141. Bolt DM, Burba DJ, Hubert JD, Pettifer GR, Hosgood GL. Evaluation of cutaneous analgesia after non-focused extracorporeal shock wave application over the 3rd metacarpal bone in horses. Can J Vet Res. 2004;68(4):288-92.
142. Orhan Z, Alper M, Akman Y, Yavuz O, Yalciner A. An experimental study on the application of extracorporeal shock waves in the treatment of tendon injuries: preliminary report. J Orthop Sci. 2001;6(6):566-70.
143. Stromberg B, Tufvesson G, Nilsson G. Effect of surgical splitting on vascular reactions in the superficial flexor tendon of the horse. Journal of the American Veterinary Medical Association. 1974;164(1):57-60.
144. Silver IA, Brown PN, Goodship AE, Lanyon LE, McCullagh KG, Perry GC, et al. A clinical and experimental study of tendon injury, healing and treatment in the horse. Equine veterinary journal Supplement. 1983(1):1-43.
145. Henninger R. Treatment of superficial digital flexor tendinitis. Veterinary Clinics of North America - Equine Practice. 1994;10(2):409-24.
146. Bramlage LR, editor. Superior check ligament desmotomy as a treatment for superficial digital flexor tendinitis: initial report. 32nd annual convention AAEP; 1986; Nashville, Tenessee.
147. Shoemaker RS, Bertone AL, Mohammad LN, Arms SW. Desmotomy of the accessory ligament of the superficial digital flexor muscle in equine cadaver limbs. Veterinary Surgery. 1991;20(4):245-52.
148. Gibson KT, Burbidge HM, Pfeiffer DU. Superficial digital flexor tendonitis in thoroughbred race horses: outcome following non-surgical treatment and superior check desmotomy. Australian veterinary journal. 1997;75(9):631-5.
149. Richardson LE, Dudhia J, Clegg PD, Smith R. Stem cells in veterinary medicine--attempts at regenerating equine tendon after injury. Trends in biotechnology. 2007;25(9):409-16. Epub 2007/08/19.
150. Maffulli N, Moller HD, Evans CH. Tendon healing: can it be optimised? British journal of sports medicine. 2002;36(5):315-6. Epub 2002/09/28.
151. Hsu C, Chang J. Clinical implications of growth factors in flexor tendon wound healing. The Journal of hand surgery. 2004;29(4):551-63. Epub 2004/07/14.
152. Bathe AP, editor. 1st World Orthopaedic Veterinary Congress of the ESVOT and VOS. World Orthopaedic Veterinary Congress; 2002; Munich: Schattauer.
153. Herthel DJ, editor. Enhanced Suspensory Ligament Healing in 100 Horses by Stem Cells and Other Bone Marrow Components. 47th Annual Convention of the American Association of Equine Practitioners; 2001; SanDiego, California.
154. Smith JJ, Ross MW, Smith RK. Anabolic effects of acellular bone marrow, platelet rich plasma, and serum on equine suspensory ligament fibroblasts in vitro. Veterinary and comparative orthopaedics and traumatology : VCOT. 2006;19(1):43-7. Epub 2006/04/06.
155. Mazzocca AD, McCarthy MB, Chowaniec DM, Cote MP, Romeo AA, Bradley JP, et al. Platelet-rich plasma differs according to preparation method and human variability. The Journal of bone and joint surgery American volume. 2012;94(4):308-16. Epub 2012/02/18.
156. Tinsley BA, Ferreira JV, Dukas AG, Mazzocca AD. Platelet-Rich Plasma Nonoperative Injection Therapy-A Review of Indications and Evidence. Oper Techn Sport Med. 2012;20(2):192-200.
157. Marx RE. Platelet-rich plasma (PRP): what is PRP and what is not PRP? Implant dentistry. 2001;10(4):225-8. Epub 2002/01/30.
158. DeLong JM, Russell RP, Mazzocca AD. Platelet-rich plasma: the PAW classification system. Arthroscopy : the journal of arthroscopic & related surgery : official publication of the Arthroscopy Association of North America and the International Arthroscopy Association. 2012;28(7):998-1009. Epub 2012/06/29.

159. Wallace SS, Froum SJ. Effect of maxillary sinus augmentation on the survival of endosseous dental implants. A systematic review. Annals of periodontology / the American Academy of Periodontology. 2003;8(1):328-43. Epub 2004/02/20.
160. Fennis JP, Stoelinga PJ, Jansen JA. Mandibular reconstruction: a histological and histomorphometric study on the use of autogenous scaffolds, particulate cortico-cancellous bone grafts and platelet rich plasma in goats. International journal of oral and maxillofacial surgery. 2004;33(1):48-55. Epub 2003/12/24.
161. Tozum TF, Demiralp B. Platelet-rich plasma: a promising innovation in dentistry. Journal. 2003;69(10):664. Epub 2003/11/13.
162. Rodriguez A, Anastassov GE, Lee H, Buchbinder D, Wettan H. Maxillary sinus augmentation with deproteinated bovine bone and platelet rich plasma with simultaneous insertion of endosseous implants. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2003;61(2):157-63. Epub 2003/03/06.
163. Marx RE. Platelet-rich plasma: evidence to support its use. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2004;62(4):489-96. Epub 2004/04/16.
164. Roldan JC, Jepsen S, Miller J, Freitag S, Rueger DC, Acil Y, et al. Bone formation in the presence of platelet-rich plasma vs. bone morphogenetic protein-7. Bone. 2004;34(1):80-90. Epub 2004/01/31.
165. Lucarelli E, Fini M, Beccheroni A, Giavaresi G, Di Bella C, Aldini NN, et al. Stromal stem cells and platelet-rich plasma improve bone allograft integration. Clinical orthopaedics and related research. 2005(435):62-8. Epub 2005/06/03.
166. Akeda K, An HS, Pichika R, Attawia M, Thonar EJ, Lenz ME, et al. Platelet-rich plasma (PRP) stimulates the extracellular matrix metabolism of porcine nucleus pulposus and anulus fibrosus cells cultured in alginate beads. Spine. 2006;31(9):959-66. Epub 2006/04/28.
167. Schnabel LV, Mohammed HO, Miller BJ, McDermott WG, Jacobson MS, Santangelo KS, et al. Platelet rich plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2007;25(2):230-40. Epub 2006/11/16.
168. Bosch G, van Schie HT, de Groot MW, Cadby JA, van de Lest CH, Barneveld A, et al. Effects of platelet-rich plasma on the quality of repair of mechanically induced core lesions in equine superficial digital flexor tendons: A placebo-controlled experimental study. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2010;28(2):211-7. Epub 2009/08/29.
169. Waselau M, Sutter WW, Genovese RL, Bertone AL. Intralesional injection of platelet-rich plasma followed by controlled exercise for treatment of midbody suspensory ligament desmitis in Standardbred racehorses. Journal of the American Veterinary Medical Association. 2008;232(10):1515-20. Epub 2008/05/16.
170. Beattie AJ, Gilbert TW, Guyot JP, Yates AJ, Badylak SF. Chemoattraction of progenitor cells by remodeling extracellular matrix scaffolds. Tissue engineering Part A. 2009;15(5):1119-25. Epub 2008/10/08.
171. Wallis T, Baxter GD, Werpy N, editors. Acellular urinary bladder matrix in a collagnease model of superficial digital flexor tendonitis in horses. 39th Annual Convention Aerican Association of Equine Practitioners; 2007; Orlando, Florida.
172. Dahlgren AL, Harvey SC, editors. Effects of autologous conditioned serum on the metabolism of normal tendon explants. 54th Annual Meeting of the Orthopaedic Research Society; 2008; San Francisco, California.
173. Easter JL, Watts AE, editors. How to Select Cases and Use Autologous Conditioned Serum to Treat Proximal Suspensory Desmitis. 60th Annual Convention American Association of Equine Practitioners; 2014; Salt Lake Coty, Utha.

174. Adzick NS, Longaker MT. Scarless fetal healing. Therapeutic implications. Annals of surgery. 1992;215(1):3-7. Epub 1992/01/01.
175. Burrington JD. Wound healing in the fetal lamb. Journal of pediatric surgery. 1971;6(5):523-8. Epub 1971/10/01.
176. Rowlatt U. Intrauterine wound healing in a 20 week human fetus. Virchows Archiv A, Pathological anatomy and histology. 1979;381(3):353-61. Epub 1979/03/23.
177. Lin KY, Posnick JC, al-Qattan MM, Vajsar J, Becker LE. Fetal nerve healing: an experimental study. Plastic and reconstructive surgery. 1994;93(7):1323-33. Epub 1994/06/01.
178. Namba RS, Meuli M, Sullivan KM, Le AX, Adzick NS. Spontaneous repair of superficial defects in articular cartilage in a fetal lamb model. The Journal of bone and joint surgery American volume. 1998;80(1):4-10. Epub 1998/02/20.
179. Slate RK, Posnick JC, Wells MD, Goldstein JA, Keeley FW, Thorner PS. Fetal tibial bone healing in utero: the effects of miniplate fixation. Plastic and reconstructive surgery. 1993;92(5):874-83. Epub 1993/10/01.
180. Weinzweig J, Panter KE, Pantaloni M, Spangenberger A, Harper JS, Lui F, et al. The fetal cleft palate: II. Scarless healing after in utero repair of a congenital model. Plastic and reconstructive surgery. 1999;104(5):1356-64. Epub 1999/10/08.
181. Weinzweig J, Panter KE, Pantaloni M, Spangenberger A, Harper JS, Lui F, et al. The fetal cleft palate: I. Characterization of a congenital model. Plastic and reconstructive surgery. 1999;103(2):419-28. Epub 1999/02/09.
182. Beredjiklian PK, Favata M, Cartmell JS, Flanagan CL, Crombleholme TM, Soslowsky LJ. Regenerative versus reparative healing in tendon: a study of biomechanical and histological properties in fetal sheep. Annals of biomedical engineering. 2003;31(10):1143-52. Epub 2003/12/03.
183. Peled ZM, Rhee SJ, Hsu M, Chang J, Krummel TM, Longaker MT. The ontogeny of scarless healing II: EGF and PDGF-B gene expression in fetal rat skin and fibroblasts as a function of gestational age. Annals of plastic surgery. 2001;47(4):417-24. Epub 2001/10/17.
184. Glass ZA, Schiele NR, Kuo CK. Informing tendon tissue engineering with embryonic development. J Biomech. 2014;47(9):1964-8.
185. Lorda-Diez CI, Montero JA, Garcia-Porrero JA, Hurle JM. Divergent differentiation of skeletal progenitors into cartilage and tendon: lessons from the embryonic limb. ACS chemical biology. 2014;9(1):72-9. Epub 2013/11/16.
186. Bosch P, Musgrave DS, Lee JY, Cummins J, Shuler T, Ghivizzani TC, et al. Osteoprogenitor cells within skeletal muscle. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2000;18(6):933-44. Epub 2001/02/24.
187. Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Experimental hematology. 2002;30(8):896-904. Epub 2002/08/06.
188. Huard J, Cao B, Qu-Petersen Z. Muscle-derived stem cells: potential for muscle regeneration. Birth defects research Part C, Embryo today : reviews. 2003;69(3):230-7. Epub 2003/12/16.
189. da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. Journal of cell science. 2006;119(Pt 11):2204-13. Epub 2006/05/11.
190. Barbero A, Ploegert S, Heberer M, Martin I. Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis and rheumatism. 2003;48(5):1315-25. Epub 2003/05/15.
191. Noth U, Osyczka AM, Tuli R, Hickok NJ, Danielson KG, Tuan RS. Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2002;20(5):1060-9. Epub 2002/10/18.
192. Yen BL, Huang HI, Chien CC, Jui HY, Ko BS, Yao M, et al. Isolation of multipotent cells from human term placenta. Stem cells. 2005;23(1):3-9. Epub 2004/12/31.

193. Salingcarnboriboon R, Yoshitake H, Tsuji K, Obinata M, Amagasa T, Nifuji A, et al. Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. Experimental cell research. 2003;287(2):289-300. Epub 2003/07/03.
194. Kryger GS, Chong AK, Costa M, Pham H, Bates SJ, Chang J. A comparison of tenocytes and mesenchymal stem cells for use in flexor tendon tissue engineering. The Journal of hand surgery. 2007;32(5):597-605. Epub 2007/05/08.
195. Goodman SA, May SA, Heinegard D, Smith RK. Tenocyte response to cyclical strain and transforming growth factor beta is dependent upon age and site of origin. Biorheology. 2004;41(5):613-28. Epub 2004/10/13.
196. Liu W, Chen B, Deng D, Xu F, Cui L, Cao Y. Repair of tendon defect with dermal fibroblast engineered tendon in a porcine model. Tissue engineering. 2006;12(4):775-88. Epub 2006/05/06.
197. Evans CE, Trail IA. Fibroblast-like cells from tendons differ from skin fibroblasts in their ability to form three-dimensional structures in vitro. Journal of hand surgery. 1998;23(5):633-41. Epub 1998/11/20.
198. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med. 2004;8(3):301-16.
199. Minguell JJ, Erices A, Conget P. Mesenchymal stem cells. Exp Biol Med. 2001;226(6):507-20.
200. Lee EH, Hui JH. The potential of stem cells in orthopaedic surgery. The Journal of bone and joint surgery British volume. 2006;88(7):841-51. Epub 2006/06/27.
201. Brederlau A, Correia AS, Anisimov SV, Elmi M, Paul G, Roybon L, et al. Transplantation of human embryonic stem cell-derived cells to a rat model of Parkinson's disease: effect of in vitro differentiation on graft survival and teratoma formation. Stem cells. 2006;24(6):1433-40. Epub 2006/03/25.
202. Nussbaum J, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, et al. Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2007;21(7):1345-57. Epub 2007/02/08.
203. Lees JG, Lim SA, Croll T, Williams G, Lui S, Cooper-White J, et al. Transplantation of 3D scaffolds seeded with human embryonic stem cells: biological features of surrogate tissue and teratoma-forming potential. Regenerative medicine. 2007;2(3):289-300. Epub 2007/05/22.
204. Prokhorova TA, Harkness LM, Frandsen U, Ditzel N, Schroder HD, Burns JS, et al. Teratoma formation by human embryonic stem cells is site dependent and enhanced by the presence of Matrigel. Stem Cells Dev. 2009;18(1):47-54. Epub 2008/04/09.
205. Hentze H, Soong PL, Wang ST, Phillips BW, Putti TC, Dunn NR. Teratoma formation by human embryonic stem cells: evaluation of essential parameters for future safety studies. Stem cell research. 2009;2(3):198-210. Epub 2009/04/28.
206. Gutierrez-Aranda I, Ramos-Mejia V, Bueno C, Munoz-Lopez M, Real PJ, Macia A, et al. Human induced pluripotent stem cells develop teratoma more efficiently and faster than human embryonic stem cells regardless the site of injection. Stem cells. 2010;28(9):1568-70. Epub 2010/07/20.
207. Kraus KH, Kirker-Head C. Mesenchymal stem cells and bone regeneration. Veterinary surgery : VS. 2006;35(3):232-42. Epub 2006/04/26.
208. Brehm W, Aklin B, Yamashita T, Rieser F, Trub T, Jakob RP, et al. Repair of superficial osteochondral defects with an autologous scaffold-free cartilage construct in a caprine model: implantation method and short-term results. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2006;14(12):1214-26. Epub 2006/07/06.
209. Wilke MM, Nydam DV, Nixon AJ. Enhanced early chondrogenesis in articular defects following arthroscopic mesenchymal stem cell implantation in an equine model. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2007;25(7):913-25. Epub 2007/04/04.

210. Nixon AJ, Dahlgren LA, Haupt JL, Yeager AE, Ward DL. Effect of adipose-derived nucleated cell fractions on tendon repair in horses with collagenase-induced tendinitis. American journal of veterinary research. 2008;69(7):928-37.
211. Richardson LE, Dudhia J, Clegg PD, Smith R. Stem cells in veterinary medicine - attempts at regenerating equine tendon after injury. Trends in biotechnology. 2007;25(9):409-16.
212. Brent AE, Braun T, Tabin CJ. Genetic analysis of interactions between the somitic muscle, cartilage and tendon cell lineages during mouse development. Development. 2005;132(3):515-28. Epub 2005/01/07.
213. Wolfman NM, Hattersley G, Cox K, Celeste AJ, Nelson R, Yamaji N, et al. Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, and 7, members of the TGF-beta gene family. The Journal of clinical investigation. 1997;100(2):321-30. Epub 1997/07/15.
214. Maeda T, Sakabe T, Sunaga A, Sakai K, Rivera AL, Keene DR, et al. Conversion of mechanical force into TGF-beta-mediated biochemical signals. Current biology : CB. 2011;21(11):933-41. Epub 2011/05/24.
215. Pryce BA, Watson SS, Murchison ND, Staverosky JA, Dunker N, Schweitzer R. Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. Development. 2009;136(8):1351-61. Epub 2009/03/24.
216. Hoffmann A, Pelled G, Turgeman G, Eberle P, Zilberman Y, Shinar H, et al. Neotendon formation induced by manipulation of the Smad8 signalling pathway in mesenchymal stem cells. Journal of Clinical Investigation. 2006;116(4):940-52.
217. Doroski DM, Levenston ME, Temenoff JS. Cyclic tensile culture promotes fibroblastic differentiation of marrow stromal cells encapsulated in poly(ethylene glycol)-based hydrogels. Tissue engineering Part A. 2010;16(11):3457-66. Epub 2010/07/30.
218. Kuo CK, Tuan RS. Mechanoactive tenogenic differentiation of human mesenchymal stem cells. Tissue engineering Part A. 2008;14(10):1615-27. Epub 2008/09/02.
219. Scott A, Danielson P, Abraham T, Fong G, Sampaio AV, Underhill TM. Mechanical force modulates scleraxis expression in bioartificial tendons. Journal of musculoskeletal & neuronal interactions. 2011;11(2):124-32. Epub 2011/06/01.
220. Sharma RI, Snedeker JG. Biochemical and biomechanical gradients for directed bone marrow stromal cell differentiation toward tendon and bone. Biomaterials. 2010;31(30):7695-704. Epub 2010/07/27.
221. Thomopoulos S, Das R, Birman V, Smith L, Ku K, Elson EL, et al. Fibrocartilage tissue engineering: the role of the stress environment on cell morphology and matrix expression. Tissue engineering Part A. 2011;17(7-8):1039-53. Epub 2010/11/26.
222. Riboh J, Chong AKS, Pham H, Longaker M, Jacobs C, Chang J. Optimization of Flexor Tendon Tissue Engineering With a Cyclic Strain Bioreactor. J Hand Surg-Am. 2008;33A(8):1388-96.
223. Lejard V, Brideau G, Blais F, Salingcarnboriboon R, Wagner G, Roehrl MH, et al. Scleraxis and NFATc regulate the expression of the pro-alpha1(I) collagen gene in tendon fibroblasts. The Journal of biological chemistry. 2007;282(24):17665-75. Epub 2007/04/14.
224. Kardon G. Muscle and tendon morphogenesis in the avian hind limb. Development. 1998;125(20):4019-32. Epub 1998/09/15.
225. Jelinsky SA, Archambault J, Li L, Seeherman H. Tendon-selective genes identified from rat and human musculoskeletal tissues. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2010;28(3):289-97. Epub 2009/09/26.
226. Young RG, Butler DL, Weber W, Caplan AI, Gordon SL, Fink DJ. Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J Orthopaed Res. 1998;16(4):406-13.
227. Awad HA, Boivin GP, Dressler MR, Smith FNL, Young RG, Butler DL. Repair of patellar tendon injuries using a cell-collagen composite. J Orthopaed Res. 2003;21(3):420-31.

228. Hankemeier S, Keus M, Zeichen J, Jagodzinski M, Barkhausen T, Bosch U, et al. Modulation of proliferation and differentiation of human bone marrow stromal cells by fibroblast growth factor 2: Potential implications for tissue engineering of tendons and ligaments. Tissue engineering. 2005;11(1-2):41-9.
229. Dyson SJ. Medical management of superficial digital flexor tendonitis: a comparative study in 219 horses (1992-2000). Equine veterinary journal. 2004;36(5):415-9.
230. Fortier LA, Smith RKW. Regenerative medicine for tendinous and ligamentous injuries of sport horses. Vet Clin N Am-Equine. 2008;24(1):191-+.
231. Soslowsky LJ, Thomopoulos S, Tun S, Flanagan CL, Keefer CC, Mastaw J, et al. Neer Award 1999. Overuse activity injures the supraspinatus tendon in an animal model: a histologic and biomechanical study. Journal of shoulder and elbow surgery / American Shoulder and Elbow Surgeons [et al]. 2000;9(2):79-84. Epub 2000/05/16.
232. Soslowsky LJ, Thomopoulos S, Esmail A, Flanagan CL, Iannotti JP, Williamson JD, 3rd, et al. Rotator cuff tendinosis in an animal model: role of extrinsic and overuse factors. Annals of biomedical engineering. 2002;30(8):1057-63. Epub 2002/11/27.
233. Perry SM, McIlhenny SE, Hoffman MC, Soslowsky LJ. Inflammatory and angiogenic mRNA levels are altered in a supraspinatus tendon overuse animal model. Journal of shoulder and elbow surgery / American Shoulder and Elbow Surgeons [et al]. 2005;14(1 Suppl S):79S-83S. Epub 2005/02/24.
234. Archambault JM, Jelinsky SA, Lake SP, Hill AA, Glaser DL, Soslowsky LJ. Rat supraspinatus tendon expresses cartilage markers with overuse. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2007;25(5):617-24. Epub 2007/02/24.
235. Scott A, Cook JL, Hart DA, Walker DC, Duronio V, Khan KM. Tenocyte responses to mechanical loading in vivo: a role for local insulin-like growth factor 1 signaling in early tendinosis in rats. Arthritis and rheumatism. 2007;56(3):871-81. Epub 2007/03/01.
236. Jelinsky SA, Lake SP, Archambault JM, Soslowsky LJ. Gene expression in rat supraspinatus tendon recovers from overuse with rest. Clinical orthopaedics and related research. 2008;466(7):1612-7. Epub 2008/05/07.
237. Glazebrook MA, Wright JR, Jr., Langman M, Stanish WD, Lee JM. Histological analysis of achilles tendons in an overuse rat model. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2008;26(6):840-6. Epub 2008/01/10.
238. Nakama LH, King KB, Abrahamsson S, Rempel DM. Evidence of tendon microtears due to cyclical loading in an in vivo tendinopathy model. J Orthopaed Res. 2005;23(5):1199-205.
239. Nakama LH, King KB, Abrahamsson S, Rempel DM. VEGF, VEGFR-1, and CTGF cell densities in tendon are increased-with cyclical loading: An in vivo tendinopathy model. J Orthopaed Res. 2006;24(3):393-400.
240. Sun HB, Li Y, Fung DT, Majeska RJ, Schaffler MB, Flatow EL. Coordinate regulation of IL-1beta and MMP-13 in rat tendons following subrupture fatigue damage. Clinical orthopaedics and related research. 2008;466(7):1555-61. Epub 2008/05/13.
241. Fung DT, Wang VM, Andarawis-Puri N, Basta-Pljakic J, Li Y, Laudier DM, et al. Early response to tendon fatigue damage accumulation in a novel in vivo model. J Biomech. 2010;43(2):274-9. Epub 2009/11/27.
242. Soslowsky LJ, Carpenter JE, DeBano CM, Banerji I, Moalli MR. Development and use of an animal model for investigations on rotator cuff disease. Journal of shoulder and elbow surgery / American Shoulder and Elbow Surgeons [et al]. 1996;5(5):383-92. Epub 1996/09/01.
243. Stone D, Green C, Rao U, Aizawa H, Yamaji T, Niyibizi C, et al. Cytokine-induced tendinitis: a preliminary study in rabbits. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 1999;17(2):168-77. Epub 1999/04/30.
244. Hsu RW, Hsu WH, Tai CL, Lee KF. Effect of hyperbaric oxygen therapy on patellar tendinopathy in a rabbit model. The Journal of trauma. 2004;57(5):1060-4. Epub 2004/12/08.

245. Dahlgren LA, Mohammed HO, Nixon AJ. Expression of insulin-like growth factor binding proteins in healing tendon lesions. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2006;24(2):183-92. Epub 2006/01/26.
246. Lui PP, Fu SC, Chan LS, Hung LK, Chan KM. Chondrocyte phenotype and ectopic ossification in collagenase-induced tendon degeneration. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 2009;57(2):91-100. Epub 2008/10/01.
247. Fu SC, Wang W, Pau HM, Wong YP, Chan KM, Rolf CG. Increased expression of transforming growth factor-beta1 in patellar tendinosis. Clinical orthopaedics and related research. 2002(400):174-83. Epub 2002/06/20.
248. Langberg H, Skovgaard D, Karamouzis M, Bulow J, Kjaer M. Metabolism and inflammatory mediators in the peritendinous space measured by microdialysis during intermittent isometric exercise in humans. The Journal of physiology. 1999;515 (Pt 3):919-27. Epub 1999/03/06.
249. Zhang J, Wang JH. Production of PGE(2) increases in tendons subjected to repetitive mechanical loading and induces differentiation of tendon stem cells into non-tenocytes. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2010;28(2):198-203. Epub 2009/08/19.
250. Khan MH, Li Z, Wang JH. Repeated exposure of tendon to prostaglandin-E2 leads to localized tendon degeneration. Clinical journal of sport medicine : official journal of the Canadian Academy of Sport Medicine. 2005;15(1):27-33. Epub 2005/01/18.
251. Sullo A, Maffulli N, Capasso G, Testa V. The effects of prolonged peritendinous administration of PGE1 to the rat Achilles tendon: a possible animal model of chronic Achilles tendinopathy. J Orthop Sci. 2001;6(4):349-57. Epub 2001/08/02.
252. van der Linden PD, van Puijenbroek EP, Feenstra J, Veld BA, Sturkenboom MC, Herings RM, et al. Tendon disorders attributed to fluoroquinolones: a study on 42 spontaneous reports in the period 1988 to 1998. Arthritis and rheumatism. 2001;45(3):235-9. Epub 2001/06/21.
253. Astrom M, Gentz CF, Nilsson P, Rausing A, Sjoberg S, Westlin N. Imaging in chronic achilles tendinopathy: a comparison of ultrasonography, magnetic resonance imaging and surgical findings in 27 histologically verified cases. Skeletal radiology. 1996;25(7):615-20. Epub 1996/10/01.
254. Movin T, Gad A, Guntner P, Foldhazy Z, Rolf C. Pathology of the Achilles tendon in association with ciprofloxacin treatment. Foot & ankle international. 1997;18(5):297-9. Epub 1997/05/01.
255. Shakibaei M, de Souza P, van Sickle D, Stahlmann R. Biochemical changes in Achilles tendon from juvenile dogs after treatment with ciprofloxacin or feeding a magnesium-deficient diet. Archives of toxicology. 2001;75(6):369-74. Epub 2001/09/26.
256. Watkins JP, Auer JA, Gay S, Morgan SJ. Healing of surgically created defects in the equine superficial digital flexor tendon: collagen-type transformation and tissue morphologic reorganization. American journal of veterinary research. 1985;46(10):2091-6. Epub 1985/10/01.
257. Bertone AL, Stashak TS, Smith FW, Norrdin RW. A comparison of repair methods for gap healing in equine flexor tendon. Veterinary surgery : VS. 1990;19(4):254-65. Epub 1990/07/01.
258. van Schie HT, Bakker EM, Cherdchutham W, Jonker AM, van de Lest CH, van Weeren PR. Monitoring of the repair process of surgically created lesions in equine superficial digital flexor tendons by use of computerized ultrasonography. American journal of veterinary research. 2009;70(1):37-48. Epub 2009/01/06.
259. Chong AK, Ang AD, Goh JC, Hui JH, Lim AY, Lee EH, et al. Bone marrow-derived mesenchymal stem cells influence early tendon-healing in a rabbit achilles tendon model. The Journal of bone and joint surgery American volume. 2007;89(1):74-81. Epub 2007/01/04.
260. Virchenko O, Fahlgren A, Rundgren M, Aspenberg P. Early Achilles tendon healing in sheep. Archives of orthopaedic and trauma surgery. 2008;128(9):1001-6. Epub 2008/07/05.
261. Schramme M, Hunter S, Campbell N, Blikslager A, Smith R. A surgical tendonitis model in horses: technique, clinical, ultrasonographic and histological characterisation. Veterinary and comparative orthopaedics and traumatology : VCOT. 2010;23(4):231-9. Epub 2010/06/30.

262. Stoll C, John T, Endres M, Rosen C, Kaps C, Kohl B, et al. Extracellular matrix expression of human tenocytes in three-dimensional air-liquid and PLGA cultures compared with tendon tissue: implications for tendon tissue engineering. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2010;28(9):1170-7. Epub 2010/02/27.
263. Schulze-Tanzil G, Mobasheri A, Clegg PD, Sendzik J, John T, Shakibaei M. Cultivation of human tenocytes in high-density culture. Histochemistry and cell biology. 2004;122(3):219-28. Epub 2004/09/01.
264. Schneider PR, Buhrmann C, Mobasheri A, Matis U, Shakibaei M. Three-dimensional high-density co-culture with primary tenocytes induces tenogenic differentiation in mesenchymal stem cells. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2011;29(9):1351-60. Epub 2011/03/26.
265. Taylor SE, Vaughan-Thomas A, Clements DN, Pinchbeck G, Macrory LC, Smith RK, et al. Gene expression markers of tendon fibroblasts in normal and diseased tissue compared to monolayer and three dimensional culture systems. BMC musculoskeletal disorders. 2009;10:27. Epub 2009/02/28.
266. Pauly S, Klatte F, Strobel C, Schmidmaier G, Greiner S, Scheibel M, et al. Characterization of tendon cell cultures of the human rotator cuff. European cells & materials. 2010;20:84-97. Epub 2010/07/28.
267. Shukunami C, Takimoto A, Oro M, Hiraki Y. Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. Developmental biology. 2006;298(1):234-47. Epub 2006/08/01.
268. Sawaguchi N, Majima T, Iwasaki N, Funakoshi T, Shimode K, Onodera T, et al. Extracellular matrix modulates expression of cell-surface proteoglycan genes in fibroblasts. Connective tissue research. 2006;47(3):141-8. Epub 2006/06/07.
269. Zhu J, Li J, Wang B, Zhang WJ, Zhou G, Cao Y, et al. The regulation of phenotype of cultured tenocytes by microgrooved surface structure. Biomaterials. 2010;31(27):6952-8. Epub 2010/07/20.
270. Qiu Y, Wang X, Zhang Y, Rout R, Carr AJ, Zhu L, et al. Development of a refined tenocyte differentiation culture technique for tendon tissue engineering. Cells, tissues, organs. 2013;197(1):27-36. Epub 2012/09/12.
271. Qiu Y, Wang X, Zhang Y, Carr AJ, Zhu L, Xia Z, et al. In vitro two-dimensional and three-dimensional tenocyte culture for tendon tissue engineering. Journal of tissue engineering and regenerative medicine. 2013. Epub 2013/09/17.
272. Wagenhauser MU, Pietschmann MF, Sievers B, Docheva D, Schieker M, Jansson V, et al. Collagen type I and decorin expression in tenocytes depend on the cell isolation method. BMC musculoskeletal disorders. 2012;13:140. Epub 2012/08/09.
273. Liu W, Watson SS, Lan Y, Keene DR, Ovitt CE, Liu H, et al. The atypical homeodomain transcription factor Mohawk controls tendon morphogenesis. Molecular and cellular biology. 2010;30(20):4797-807. Epub 2010/08/11.
274. Ito Y, Toriuchi N, Yoshitaka T, Ueno-Kudoh H, Sato T, Yokoyama S, et al. The Mohawk homeobox gene is a critical regulator of tendon differentiation. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(23):10538-42. Epub 2010/05/26.
275. Lejard V, Blais F, Guerquin MJ, Bonnet A, Bonnin MA, Havis E, et al. EGR1 and EGR2 involvement in vertebrate tendon differentiation. The Journal of biological chemistry. 2011;286(7):5855-67. Epub 2010/12/22.
276. Yamamoto-Shiraishi Y, Kuroiwa A. Wnt and BMP signaling cooperate with Hox in the control of Six2 expression in limb tendon precursor. Developmental biology. 2013;377(2):363-74. Epub 2013/03/19.
277. Pearse RV, 2nd, Esshaki D, Tabin CJ, Murray MM. Genome-wide expression analysis of intra- and extraarticular connective tissue. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2009;27(4):427-34. Epub 2008/10/31.

278. Murchison ND, Price BA, Conner DA, Keene DR, Olson EN, Tabin CJ, et al. Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. Development. 2007;134(14):2697-708. Epub 2007/06/15.
279. Schweitzer R, Chyung JH, Murtaugh LC, Brent AE, Rosen V, Olson EN, et al. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. Development. 2001;128(19):3855-66. Epub 2001/10/05.
280. Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nature medicine. 2007;13(10):1219-27. Epub 2007/09/11.
281. Cserjesi P, Brown D, Ligon KL, Lyons GE, Copeland NG, Gilbert DJ, et al. Scleraxis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. Development. 1995;121(4):1099-110. Epub 1995/04/01.
282. Brown D, Wagner D, Li X, Richardson JA, Olson EN. Dual role of the basic helix-loop-helix transcription factor scleraxis in mesoderm formation and chondrogenesis during mouse embryogenesis. Development. 1999;126(19):4317-29. Epub 1999/09/08.
283. Brent AE, Schweitzer R, Tabin CJ. A somitic compartment of tendon progenitors. Cell. 2003;113(2):235-48. Epub 2003/04/23.
284. Smith TG, Sweetman D, Patterson M, Keyse SM, Munsterberg A. Feedback interactions between MKP3 and ERK MAP kinase control scleraxis expression and the specification of rib progenitors in the developing chick somite. Development. 2005;132(6):1305-14. Epub 2005/02/18.
285. Edom-Vovard F, Schuler B, Bonnin MA, Teillet MA, Duprez D. Fgf4 positively regulates scleraxis and tenascin expression in chick limb tendons. Developmental biology. 2002;247(2):351-66. Epub 2002/06/28.
286. Espira L, Lamoureux L, Jones SC, Gerard RD, Dixon IM, Czubryt MP. The basic helix-loop-helix transcription factor scleraxis regulates fibroblast collagen synthesis. Journal of molecular and cellular cardiology. 2009;47(2):188-95. Epub 2009/04/14.
287. Shukunami C, Oshima Y, Hiraki Y. Molecular cloning of tenomodulin, a novel chondromodulin-I related gene. Biochemical and biophysical research communications. 2001;280(5):1323-7. Epub 2001/02/13.
288. Docheva D, Hunziker EB, Fassler R, Brandau O. Tenomodulin is necessary for tenocyte proliferation and tendon maturation. Molecular and cellular biology. 2005;25(2):699-705. Epub 2005/01/06.
289. Oshima Y, Sato K, Tashiro F, Miyazaki J, Nishida K, Hiraki Y, et al. Anti-angiogenic action of the C-terminal domain of tenomodulin that shares homology with chondromodulin-I. Journal of cell science. 2004;117(Pt 13):2731-44. Epub 2004/05/20.
290. Brandau O, Meindl A, Fassler R, Aszodi A. A novel gene, tendin, is strongly expressed in tendons and ligaments and shows high homology with chondromodulin-I. Developmental dynamics : an official publication of the American Association of Anatomists. 2001;221(1):72-80. Epub 2001/05/18.
291. Oshima Y, Shukunami C, Honda J, Nishida K, Tashiro F, Miyazaki J, et al. Expression and localization of tenomodulin, a transmembrane type chondromodulin-I-related angiogenesis inhibitor, in mouse eyes. Investigative ophthalmology & visual science. 2003;44(5):1814-23. Epub 2003/04/26.
292. Pisani DF, Pierson PM, Massoudi A, Leclerc L, Chopard A, Marini JF, et al. Myodulin is a novel potential angiogenic factor in skeletal muscle. Experimental cell research. 2004;292(1):40-50. Epub 2004/01/15.
293. Yamana K, Wada H, Takahashi Y, Sato H, Kasahara Y, Kiyoki M. Molecular cloning and characterization of CHM1L, a novel membrane molecule similar to chondromodulin-I. Biochemical and biophysical research communications. 2001;280(4):1101-6. Epub 2001/02/13.

294. Ros MA, Rivero FB, Hinchliffe JR, Hurle JM. Immunohistological and ultrastructural study of the developing tendons of the avian foot. Anatomy and embryology. 1995;192(6):483-96. Epub 1995/12/01.
295. Mehr D, Pardubsky PD, Martin JA, Buckwalter JA. Tenascin-C in tendon regions subjected to compression. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2000;18(4):537-45. Epub 2000/10/29.
296. Tucker RP, Spring J, Baumgartner S, Martin D, Hagios C, Poss PM, et al. Novel tenascin variants with a distinctive pattern of expression in the avian embryo. Development. 1994;120(3):637-47. Epub 1994/03/01.
297. Jarvinen TA, Kannus P, Jarvinen TL, Jozsa L, Kalimo H, Jarvinen M. Tenascin-C in the pathobiology and healing process of musculoskeletal tissue injury. Scandinavian journal of medicine & science in sports. 2000;10(6):376-82. Epub 2000/11/21.
298. Kannus P, Jozsa L, Jarvinen TA, Jarvinen TL, Kvist M, Natri A, et al. Location and distribution of non-collagenous matrix proteins in musculoskeletal tissues of rat. The Histochemical journal. 1998;30(11):799-810. Epub 1999/02/13.
299. Oberhauser AF, Marszalek PE, Erickson HP, Fernandez JM. The molecular elasticity of the extracellular matrix protein tenascin. Nature. 1998;393(6681):181-5. Epub 1998/05/29.
300. Chiquet-Ehrismann R, Tucker RP. Connective tissues: signalling by tenascins. The international journal of biochemistry & cell biology. 2004;36(6):1085-9. Epub 2004/04/20.
301. de Palma L, Marinelli M, Meme L, Pavan M. Immunohistochemistry of the enthesis organ of the human Achilles tendon. Foot & ankle international. 2004;25(6):414-8. Epub 2004/06/25.
302. Vogel KG, Keller EJ, Lenhoff RJ, Campbell K, Koob TJ. Proteoglycan synthesis by fibroblast cultures initiated from regions of adult bovine tendon subjected to different mechanical forces. European journal of cell biology. 1986;41(1):102-12. Epub 1986/06/01.
303. Riley GP, Harrall RL, Cawston TE, Hazleman BL, Mackie EJ. Tenascin-C and human tendon degeneration. The American journal of pathology. 1996;149(3):933-43. Epub 1996/09/01.
304. Mackie EJ, Ramsey S. Expression of tenascin in joint-associated tissues during development and postnatal growth. Journal of anatomy. 1996;188 (Pt 1):157-65. Epub 1996/02/01.
305. Riley G. Tendinopathy--from basic science to treatment. Nature clinical practice Rheumatology. 2008;4(2):82-9. Epub 2008/02/01.
306. Butler DL, Juncosa-Melvin N, Boivin GP, Galloway MT, Shearn JT, Gooch C, et al. Functional tissue engineering for tendon repair: A multidisciplinary strategy using mesenchymal stem cells, bioscaffolds, and mechanical stimulation. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2008;26(1):1-9. Epub 2007/08/07.
307. Domm C, Schunke M, Christesen K, Kurz B. Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2002;10(1):13-22. Epub 2002/02/14.
308. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell. 1982;30(1):215-24. Epub 1982/08/01.
309. Mallein-Gerin F, Garrone R, van der Rest M. Proteoglycan and collagen synthesis are correlated with actin organization in dedifferentiating chondrocytes. European journal of cell biology. 1991;56(2):364-73. Epub 1991/12/01.
310. Schwarz R, Colarusso L, Doty P. Maintenance of differentiation in primary cultures of avian tendon cells. Experimental cell research. 1976;102(1):63-71. Epub 1976/10/01.
311. Bernard-Beaubois K, Hecquet C, Houcine O, Hayem G, Adolphe M. Culture and characterization of juvenile rabbit tenocytes. Cell biology and toxicology. 1997;13(2):103-13. Epub 1997/02/01.
312. Yao L, Bestwick CS, Bestwick LA, Maffulli N, Aspden RM. Phenotypic drift in human tenocyte culture. Tissue engineering. 2006;12(7):1843-9. Epub 2006/08/08.

313. Lavagnino M, Arnoczky SP. In vitro alterations in cytoskeletal tensional homeostasis control gene expression in tendon cells. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2005;23(5):1211-8. Epub 2005/05/24.
314. Banes AJ, Weinhold P, Yang X, Tsuzaki M, Bynum D, Bottlang M, et al. Gap junctions regulate responses of tendon cells ex vivo to mechanical loading. Clinical orthopaedics and related research. 1999(367 Suppl):S356-70. Epub 1999/11/05.
315. Vondermark K, Gauss V, Vondermark H, Muller P. Relationship between Cell-Shape and Type of Collagen Synthesized as Chondrocytes Lose Their Cartilage Phenotype in Culture. Nature. 1977;267(5611):531-2.
316. Lincoln J, Lange AW, Yutzey KE. Hearts and bones: shared regulatory mechanisms in heart valve, cartilage, tendon, and bone development. Developmental biology. 2006;294(2):292-302. Epub 2006/04/29.
317. Rosenblatt JD, Parry DJ, Partridge TA. Phenotype of adult mouse muscle myoblasts reflects their fiber type of origin. Differentiation; research in biological diversity. 1996;60(1):39-45. Epub 1996/03/01.
318. Garvin J, Qi J, Maloney M, Banes AJ. Novel system for engineering bioartificial tendons and application of mechanical load. Tissue engineering. 2003;9(5):967-79. Epub 2003/11/25.
319. Hynes RO. Cell adhesion: old and new questions. Trends in cell biology. 1999;9(12):M33-7. Epub 1999/12/28.
320. Kelm JM, Fussenegger M. Microscale tissue engineering using gravity-enforced cell assembly. Trends in biotechnology. 2004;22(4):195-202.
321. Kelm JM, Ehler E, Nielsen LK, Schlatter S, Perriard JC, Fussenegger M. Design of artificial myocardial microtissues. Tissue engineering. 2004;10(1-2):201-14. Epub 2004/03/11.
322. Kelm JM, Diaz Sanchez-Bustamante C, Ehler E, Hoerstrup SP, Djonov V, Ittner L, et al. VEGF profiling and angiogenesis in human microtissues. Journal of biotechnology. 2005;118(2):213-29. Epub 2005/06/14.
323. Kelm JM, Ittner LM, Born W, Djonov V, Fussenegger M. Self-assembly of sensory neurons into ganglia-like microtissues. Journal of biotechnology. 2006;121(1):86-101. Epub 2005/09/08.
324. Kelm JM, Djonov V, Hoerstrup SP, Guenter CI, Ittner LM, Greve F, et al. Tissue-transplant fusion and vascularization of myocardial microtissues and macrotissues implanted into chicken embryos and rats. Tissue engineering. 2006;12(9):2541-53. Epub 2006/09/26.
325. Mirsaidi A, Tiaden AN, Richards PJ. Preparation and osteogenic differentiation of scaffold-free mouse adipose-derived stromal cell microtissue spheroids (ASC-MT). Current protocols in stem cell biology. 2013;27:Unit 2B 5. Epub 2014/02/11.
326. Mirsaidi A, Genelin K, Vetsch JR, Stanger S, Theiss F, Lindtner RA, et al. Therapeutic potential of adipose-derived stromal cells in age-related osteoporosis. Biomaterials. 2014;35(26):7326-35.
327. Edom-Vovard F, Duprez D. Signals regulating tendon formation during chick embryonic development. Developmental dynamics : an official publication of the American Association of Anatomists. 2004;229(3):449-57. Epub 2004/03/03.
328. Seo HS, Serra R. Deletion of Tgfbr2 in Prx1-cre expressing mesenchyme results in defects in development of the long bones and joints. Developmental biology. 2007;310(2):304-16. Epub 2007/09/08.
329. Lorda-Diez CI, Montero JA, Choe S, Garcia-Porrero JA, Hurle JM. Ligand- and stage-dependent divergent functions of BMP signaling in the differentiation of embryonic skeletogenic progenitors in vitro. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2014;29(3):735-48. Epub 2013/09/17.
330. Macias D, Ganan Y, Sampath TK, Piedra ME, Ros MA, Hurle JM. Role of BMP-2 and OP-1 (BMP-7) in programmed cell death and skeletogenesis during chick limb development. Development. 1997;124(6):1109-17. Epub 1997/03/01.

331. Lorda-Diez CI, Montero JA, Rodriguez-Leon J, Garcia-Porrero JA, Hurle JM. Expression and functional study of extracellular BMP antagonists during the morphogenesis of the digits and their associated connective tissues. PloS one. 2013;8(4):e60423. Epub 2013/04/11.
332. Mikic B, Schalet BJ, Clark RT, Gaschen V, Hunziker EB. GDF-5 deficiency in mice alters the ultrastructure, mechanical properties and composition of the Achilles tendon. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2001;19(3):365-71. Epub 2001/06/12.
333. Mikic B, Bierwert L, Tsou D. Achilles tendon characterization in GDF-7 deficient mice. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2006;24(4):831-41. Epub 2006/03/04.
334. Mikic B, Rossmeier K, Bierwert L. Identification of a tendon phenotype in GDF6 deficient mice. Anatomical record. 2009;292(3):396-400. Epub 2009/02/28.
335. Violini S, Ramelli P, Pisani LF, Gorni C, Mariani P. Horse bone marrow mesenchymal stem cells express embryo stem cell markers and show the ability for tenogenic differentiation by in vitro exposure to BMP-12. BMC cell biology. 2009;10:29. Epub 2009/04/23.
336. Wang QW, Chen ZL, Piao YJ. Mesenchymal stem cells differentiate into tenocytes by bone morphogenetic protein (BMP) 12 gene transfer. Journal of bioscience and bioengineering. 2005;100(4):418-22. Epub 2005/11/29.
337. Keller TC, Hogan MV, Kesturu G, James R, Balian G, Chhabra AB. Growth/differentiation factor-5 modulates the synthesis and expression of extracellular matrix and cell-adhesion-related molecules of rat Achilles tendon fibroblasts. Connective tissue research. 2011;52(4):353-64. Epub 2011/01/22.
338. Park A, Hogan MV, Kesturu GS, James R, Balian G, Chhabra AB. Adipose-derived mesenchymal stem cells treated with growth differentiation factor-5 express tendon-specific markers. Tissue engineering Part A. 2010;16(9):2941-51. Epub 2010/06/26.
339. James R, Kumbar SG, Laurencin CT, Balian G, Chhabra AB. Tendon tissue engineering: adipose-derived stem cell and GDF-5 mediated regeneration using electrospun matrix systems. Biomedical materials. 2011;6(2):025011. Epub 2011/03/26.
340. Yeh LC, Tsai AD, Lee JC. Bone morphogenetic protein-7 regulates differentially the mRNA expression of bone morphogenetic proteins and their receptors in rat achilles and patellar tendon cell cultures. Journal of cellular biochemistry. 2008;104(6):2107-22. Epub 2008/04/09.
341. Caliari SR, Harley BA. Composite growth factor supplementation strategies to enhance tenocyte bioactivity in aligned collagen-GAG scaffolds. Tissue engineering Part A. 2013;19(9-10):1100-12. Epub 2012/11/20.
342. Huisman E, Lu A, McCormack RG, Scott A. Enhanced collagen type I synthesis by human tenocytes subjected to periodic in vitro mechanical stimulation. BMC musculoskeletal disorders. 2014;15:386. Epub 2014/11/22.
343. Raabe O, Shell K, Fietz D, Freitag C, Ohrndorf A, Christ HJ, et al. Tenogenic differentiation of equine adipose-tissue-derived stem cells under the influence of tensile strain, growth differentiation factors and various oxygen tensions. Cell and tissue research. 2013;352(3):509-21. Epub 2013/02/23.
344. Screen HR, Shelton JC, Chhaya VH, Kayser MV, Bader DL, Lee DA. The influence of noncollagenous matrix components on the micromechanical environment of tendon fascicles. Annals of biomedical engineering. 2005;33(8):1090-9. Epub 2005/09/01.
345. Wall ME, Weinhold PS, Siu T, Brown TD, Banes AJ. Comparison of cellular strain with applied substrate strain in vitro. J Biomech. 2007;40(1):173-81. Epub 2006/01/13.
346. Kelm JM, Lorber V, Snedeker JG, Schmidt D, Broggini-Tenzer A, Weisstanner M, et al. A novel concept for scaffold-free vessel tissue engineering: Self-assembly of microtissue building blocks. Journal of biotechnology. 2010;148(1):46-55.

347. Hohlfeld J, de Buys Roessingh A, Hirt-Burri N, Chaubert P, Gerber S, Scaletta C, et al. Tissue engineered fetal skin constructs for paediatric burns. Lancet. 2005;366(9488):840-2. Epub 2005/09/06.
348. Hirt-Burri N, de Buys Roessingh AS, Scaletta C, Gerber S, Pioletti DP, Applegate LA, et al. Human muscular fetal cells: a potential cell source for muscular therapies. Pediatric surgery international. 2008;24(1):37-47. Epub 2007/10/27.
349. Ramelet AA, Hirt-Burri N, Raffoul W, Scaletta C, Pioletti DP, Offord E, et al. Chronic wound healing by fetal cell therapy may be explained by differential gene profiling observed in fetal versus old skin cells. Experimental gerontology. 2009;44(3):208-18. Epub 2008/12/04.
350. Hirt-Burri N, Ramelet AA, Raffoul W, de Buys Roessingh A, Scaletta C, Pioletti D, et al. Biologicals and fetal cell therapy for wound and scar management. ISRN dermatology. 2011;2011:549870. Epub 2012/03/01.
351. Grognuz A, Scaletta C, Farron A, Raffoul W, Applegate LA. Human Fetal Progenitor Tenocytes for Regenerative Medicine. Cell Transplant. 2015. Epub 2015/06/26.
352. Rooney P, Walker D, Grant ME, McClure J. Cartilage and bone formation in repairing Achilles tendons within diffusion chambers: evidence for tendon-cartilage and cartilage-bone conversion in vivo. The Journal of pathology. 1993;169(3):375-81. Epub 1993/03/01.
353. Ehlers TW, Vogel KG. Proteoglycan synthesis by fibroblasts from different regions of bovine tendon cultured in alginate beads. Comparative biochemistry and physiology Part A, Molecular & integrative physiology. 1998;121(4):355-63. Epub 1999/02/27.
354. Takimoto A, Oro M, Hiraki Y, Shukunami C. Direct conversion of tenocytes into chondrocytes by Sox9. Experimental cell research. 2012;318(13):1492-507. Epub 2012/04/19.
355. Klein MB, Pham H, Yalamanchi N, Chang J. Flexor tendon wound healing in vitro: the effect of lactate on tendon cell proliferation and collagen production. The Journal of hand surgery. 2001;26(5):847-54. Epub 2001/09/19.
356. Martin JA, Mehr D, Pardubsky PD, Buckwalter JA. The role of tenascin-C in adaptation of tendons to compressive loading. Biorheology. 2003;40(1-3):321-9. Epub 2002/11/28.
357. Lorda-Diez CI, Montero JA, Martinez-Cue C, Garcia-Porrero JA, Hurle JM. Transforming growth factors beta coordinate cartilage and tendon differentiation in the developing limb mesenchyme. The Journal of biological chemistry. 2009;284(43):29988-96. Epub 2009/09/01.
358. Schwarz RI, Bissell MJ. Dependence of the differentiated state on the cellular environment: modulation of collagen synthesis in tendon cells. Proceedings of the National Academy of Sciences of the United States of America. 1977;74(10):4453-7. Epub 1977/10/01.
359. Mazzocca AD, McCarthy MB, Chowaniec D, Cote MP, Judson CH, Apostolakos J, et al. Bone marrow-derived mesenchymal stem cells obtained during arthroscopic rotator cuff repair surgery show potential for tendon cell differentiation after treatment with insulin. Arthroscopy : the journal of arthroscopic & related surgery : official publication of the Arthroscopy Association of North America and the International Arthroscopy Association. 2011;27(11):1459-71. Epub 2011/10/08.
360. Awad HA, Butler DL, Harris MT, Ibrahim RE, Wu Y, Young RG, et al. In vitro characterization of mesenchymal stem cell-seeded collagen scaffolds for tendon repair: effects of initial seeding density on contraction kinetics. Journal of biomedical materials research. 2000;51(2):233-40. Epub 2000/05/29.

9 Acknowledgements

I have to express my deep gratitude to PD Dr. Peter J. Richards for taking over the position of my Thesis advisor after Jens Kelm went into industry business. Pete had to delve into a project far away from his own research focus, planned by somebody else, being underway for more than a year, and already revealing major difficulties. Due to his conscientiousness, general scientific interest and knowledge he was able to lead the project in a successful direction. Special credit is due to him for always being patient with a clinician spending more time in the clinics than in the lab and in particular, for his continuing support after circumstances changed.

I would also like to sincerely thank Prof. Jess Snedeker for being the co-referee of my thesis and for his technical assistance and scientific input.

Special thanks are also due to Brigitte von Rechenberg, who has already played an important role in my professional career for a long time, not only during the time of my dissertation in her Musculoskeletal Research Unit, but also during my time in clinics. It was her, believing in me and providing me with the necessary contacts to realize this project. Moreover, it was her support, which helped me to obtain the financial background for this study. I highly appreciate her enthusiasm, daily drive and her ability to belief in someone.

I would also like to thank Ali Mirsaidi for his unreplaceable helpfulness with the lab work, a companion in misfortune, as he also had to deal with microtissues.

It was also always a great pleasure to work with all other people at the CABMM, with special thanks to Silke Kalchofner-Mark and Marina Klawitter.

If science may be compared to a playground reality catches up when it comes to financing a research project. Therefore, I would like to express my deep gratitude to the “Stiftung Forschung für das Pferd” for financial support, which enabled me to perform the present study.

I would also like to thank the Graduate School for Cellular and Biomedical Sciences of the University Bern for accepting me as a PhD student, providing the framework requirements to perform this PhD, as well as for their patience with a clinician to send in his thesis.

Renate, my beloved wife, clearly deserves my deepest gratitude, as she is the person, who had to suffer most during the time of my PhD. I wouldn´t have if I had known before - but it´s curiosity arising from a gap in knowledge, which drives one to explore.

10 Declaration of Originality

Declaration of Originality

Last name, first name: Theiss, Felix

Matriculation number: 00-729-889

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Zurich, August 31, 2015

Signature

This PhD-Thesis is lovingly dedicated to my mother, Margrit Theiss; † 22nd September 2015